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**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

**03008909.8**

**PRIORITY  
DOCUMENT**

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**R C van Dijk**



Anmeldung Nr.:  
Application no.: 03008909.8  
Demande no:

Anmeldetag:  
Date of filing: 16.04.03  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
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Use of genes for increasing the oil content in plants

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s)  
revendiquée(s)

Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL  
PT RO SE SI SK TR LI

## Use of genes for increasing the oil content in plants

### Description

5 The invention relates to the use of genes that when expressed will increase the total amount of oil (i.e. triacylglycerols, monoacylglycerols, fatty acids) that is produced in transgenic organisms.

10 More specifically this invention describes the identification of genes encoding oil synthesis enhancing proteins (OEP).

In a first embodiment, this invention is directed to the protein comprising an amino acid sequence as set forth in SEQ ID NO: 2 or a functional fragment, derivative, variant, or ortologue thereof.

15 The present invention further includes the nucleotide sequence as set forth in SEQ ID NO: 1, as well as portions of the genomic sequence, the cDNA sequence, allelic variants, synthetic variants and mutants thereof. This includes sequences that are to be used as probes, vectors for transformation or cloning intermediates.

20 SEQ ID NO : 2 is the deduced amino acid sequence from the open reading frame YPR140w in SEQ ID NO : 1.

25 In a second embodiment, this invention is directed to the protein comprising an amino acid sequence as set forth in SEQ ID NO: 4 or a functional fragment, derivative, variant, or ortologue thereof.

SEQ ID NO : 4 is the deduced amino acid sequence from the open reading frame At140.1 in SEQ ID NO : 3.

30 In a third embodiment, this invention is directed to the protein comprising an amino acid sequence as set forth in SEQ ID NO: 6 or a functional fragment, derivative, variant, or ortologue thereof.

35 SEQ ID NO : 6 is the deduced amino acid sequence from the open reading frame At140.2 in SEQ ID NO : 5.

40 Another aspect of the present invention relates to those polypeptides, which have at least 60% identity to SEQ ID NO: 2, SEQ ID NO : 4 or SEQ ID NO : 6.

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The invention furthermore relates to expression constructs for expressing YPR140w, At140.1 or At140.2 in plants, preferably in plant seeds, transgenic plants expressing YPR140w, At140.1 or At140.2 and to the use of said transgenic plants for the production of food, feed, seed, pharmaceuticals or fine chemicals, in particular for the production of oils.

In oil crops like rape, sunflower, oil palm etc., the oil (i.e. triacylglycerols) is the most valuable product of the seeds or fruits and other compounds such as starch, protein and fiber is regarded as by-products with less value. Enhancing the quantity of oil per weight basis at the expense of other compounds in oil crops would therefore increase the value of the crop. If proteins that promote the allocation of reduced carbon into the production of oil can be up regulated by overexpression, the cells will accumulate more oil at the expense of other products. This approach could not only be used to increase the oil content in already high oil producing organisms such as oil crops, they could also lead to significant oil production in moderate or low oil containing crops such as soy, oat, maize, potato, sugar beats, and turnips as well as in microorganisms.

Increasing the oil content in plants and, in particular, in plant seeds is of great interest for traditional and modern plant breeding and in particular for plant biotechnology.

Owing to the increasing consumption of vegetable oils for nutrition or industrial applications, possibilities of increasing or modifying vegetable oils are increasingly the subject of current research (for example Töpfer et al. (1995) Science 268:681-686). Its aim is in particular increasing the fatty acid content in seed oils.

The fatty acids which can be obtained from the vegetable oils are also of particular interest. They are employed, for example, as bases for plasticizers, lubricants, surfactants, cosmetics and the like and are employed as valuable bases in the food and feed industries. Thus, for example, it is of particular interest to provide rapeseed oils with fatty acids with medium chain length since these are in demand in particular in the production of surfactants. With regard to medical ramifications, the long chain fatty acids (C18 and longer) found in many seed oils have been linked to reductions in hypercholesterolemia and other clinical disorders related to coronary heart disease (Brenner 1976, Adv. Exp. Med. Biol. 83:85-101). Therefore, consumption of a plant having increased levels of these types of fatty acids may reduce the risk of heart disease. Enhanced levels of seed oil content also increase large-scale production of seed oils and thereby reduce the cost of these oils.

The targeted modulation of plant metabolic pathways by recombinant methods allows the modification of the plant metabolism in an advantageous manner which, when using traditional breeding methods, could only be achieved after a complicated

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procedure or not at all. Thus, unusual fatty acids, for example specific polyunsaturated fatty acids, are only synthesized in certain plants or not at all in plants and can therefore only be produced by expressing the relevant gene in transgenic plants (for example Millar et al. (2000) Trends Plant Sci 5:95-101).

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Triacylglycerides, diacylglycerides, monoacylglycerides and other lipids are synthesized from fatty acids. Fatty acid biosynthesis and triacylglyceride biosynthesis can be considered as separate biosynthetic pathways owing to the compartmentalization, but as a single biosynthetic pathway in view of the end product. Lipid synthesis can be divided into two part-mechanisms, one which might be termed "prokaryotic" and another which may be termed "eukaryotic" (Browse et al. (1986) Biochemical J 235:25-31; Ohlrogge & Browse (1995) Plant Cell 7:957-970). The prokaryotic mechanism is localized in the plastids and encompasses the biosynthesis of the free fatty acids which are exported into the cytosol, where they enter the eukaryotic mechanism in the form of fatty acid acyl-CoA esters. In this pathway the fatty acids are esterified by glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase to the sn-1 and sn-2 positions of glycerol-3-phosphate, respectively, to yield phosphatidic acid (PA). The PA is the precursor for other polar and neutral lipids, the latter being formed in the Kennedy pathway (Voelker 1996, Genetic Engineering ed.:Setlow 18:111-113; Shanklin & Cahoon 1998, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:611-641; Frentzen 1998, Lipids 100:161-166; Millar et al. 2000, Trends Plant Sci. 5:95-101).

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Patent WO 00/18889 describes nucleic acid sequences encoding for acyltransferase related proteins, wherein said acyltransferase-like protein is active in the transfer of a fatty acyl group from a fatty acyl donor to fatty acyl acceptor and the use of such sequences to provide transgenic plants capable of producing modified lipid content and composition based on sequence predictions only. Biochemical data supporting these claims is lacking so far.

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The initial step of lipid production, the transfer of a fatty acid from an acyl-CoA to glycerol-3-phosphate was shown to be in part but not exclusively performed by two yeast genes, Gat1 and Gat2 (Zeng andf Zou 2001, J Biol Chem. 276(45):41710-6). Patent WO 02/08391 relates to DNA sequences encoding these two novel glycerol-3-phosphate and dihydroxy acetone phosphate acyltransferase enzymes and methods for utilizing said polynucleotides for alteration of lipid content in higher cells, in particular plant cells. However, no counterparts from plants could be found for Gat1 or Gat2.

40

The last step in the synthesis of triacylglycerols has been shown to occur by two different enzymatic reactions, an acyl-CoA dependent reaction catalyzed by an acyl-CoA : diacylglycerol acyltransferase (Cases, S. et al., (1998) Proc. Natl. Acad. Sci.,

USA 95, 13018-13023.; Lardizabal, et al., 2001) and the acyl-CoA independent reaction catalyzed by an phospholipid : diacylglycerol acyltransferase (Dahlgvist, et al., 2000). Two unrelated gene families encoding acyl-CoA : diacylglycerol acyltransferases have been identified in plants, animals and yeast, whereas the gene family  
5 encoding the acyl-CoA independent enzyme has been identified in yeast but not in plants or animals. In yeast, a total of four genes (are1, are2, lro1, dga1) belong to these three gene families, and they are the only genes known to contribute directly to triacylglycerol synthesis. Thus, no synthesis of triacylglycerol could be detected in yeast cells where all four genes were disrupted.

10 It is an object of the present invention to provide additional methods for increasing the oil content in plants.

We have found that this object is achieved by the present invention.

15 In the present invention we show, that another gene is present in yeast and moreover in plants, which enhances the amount of triacylglycerol and fatty acids that accumulates in wild type yeast.

20 A first subject matter of the invention comprises a method of increasing the total oil content in a plant organism or a tissue, organ, part, cell or propagation material thereof, comprising

25 a) the transgenic expression of SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5 in said plant organism or in a tissue, organ, part, cell or propagation material thereof, and

30 b) the selection of plant organisms in which - in contrast to or comparison with the starting organism - the total oil content in said plant organism or in a tissue, organ, part, cell or propagation material thereof is increased.

Other proteins resulting in the same effect as the proteins set forth in SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 (OEPs) are obtainable from the specific sequences provided herein. Furthermore, it will be apparent that one  
35 can obtain natural and synthetic OEPs, including those with modified amino acid sequences and starting materials for synthetic-protein modeling from the exemplified OEPs and from OEPs which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences that have been mutated, truncated, increased and the like, whether such sequences were partially  
40 or wholly synthesized.

Further, the nucleic acid probes (DNA or RNA) derived from the SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5 of the present invention can be used to screen and recover homologous or related sequences from a variety of plant and microbial sources.

The over-expression of SEQ ID NO : 3 or SEQ ID NO : 5 in yeast or plants increases the content in total lipids and enhances the amount of triacylglycerol and fatty acids that accumulates compared to wildtype yeasts or plants. The amount of polar lipids remained unchanged but a reduced amount of palmitic acid was observed. On the other hand the amount of oleic acids was increased.

The present invention can be characterized by the following aspects:

**Example 1**

shows the reduction of GPAT activity in yeast cells lacking the YPR140w gene.

**Example 2**

shows the increased incorporation of glycerol-3-phosphate into lipids in crude extracts of yeast over expressing the YPR140w gene.

**Example 3**

shows the increased accumulation of triacylglycerol in yeast cells expressing the YPR140w gene from a strong promoter.

**Example 4**

describes the identification of genes homologous to YPR140w in plants.

**Example 5**

shows the increased accumulation of triacylglycerol in yeast cells expressing the At140.1 or At140.2 gene from a strong promoter.

**Example 6**

shows a significantly higher total oil content in the seeds of transgenic plant lines with increased expression of the At140.1 or At140.2 gene.

The invention can furthermore be characterized by:

5 use of the nucleic acid sequences SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5 encoding a protein SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 that enhances the production of triacylglycerol (TAG);

10 genetic transformation of an oil-producing organism with one of said sequence in order to be expressed in this organism, resulting in an active protein that increases the oil content of the organism.

The nucleic acid sequence is derived from the sequence shown in SEQ ID NO : 1 from the *Saccharomyces cerevisiae* YPR140w gene (genomic clone or cDNA), SEQ ID NO: 3 or SEQ ID NO: 5 from *Arabidopsis thaliana* or from a nucleic acid sequence or cDNA that contains a nucleotide sequence coding for a protein with an amino acid sequence that is 60% or more identical to the amino acid sequences as presented in SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

20 The gene products, which we refer to as an oil synthesis enhancing protein (OEP) is most likely not itself catalyzing the synthesis of TAG, but its presence elevates the amount of TAG synthesized.

25 Furthermore the OEP as characterised by the protein sequence SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 results in an increased incorporation of (<sup>14</sup>C) glycerol-3-phosphate into lipids as described in example 3 and 5.

The instant invention pertains to a gene construct comprising a said nucleotide sequence SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 of the instant invention, which is operably linked to a heterologous nucleic acid.

30 The term operably linked means a serial organization e.g. of a promoter, coding sequence, terminator and/or further regulatory elements whereby each element can fulfill its original function during expression of the nucleotide sequence.

35 Further, a vector comprising the said nucleotide sequence SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 of the instant invention is contemplated in the instant invention. This includes also an expression vector which can harbor a selectable marker gene and/or nucleotide sequences for the replication in a host cell and/or the integration into the genome of the host cell.

40 Furthermore, this invention relates to a method for producing an OEP encoded by one of the nucleic acid sequences SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 in a host



cell or progeny thereof including genetically engineered oil seeds, yeast and moulds or any other oil-accumulating organism, via the expression of a construct in the cell. Of particular interest is the expression of the nucleotide sequences of the present invention from transcription initiation regions that are preferentially expressed in plant seed tissues. It is further contemplated that an artificial gene sequence encoding an OEP may be synthesized, especially to provide plant-preferred codons. Transgenic cells containing an OEP as a result of the expression of an OEP encoding sequence are also contemplated within the scope of the invention.

- 10 Further, the invention pertains a transgenic cell or organism containing a said nucleotide sequence and/or a said gene construct and/or a said vector. The object of the instant invention is further a transgenic cell or organism which is an eucaryotic cell or organism. Preferably, the transgenic cell or organism is a yeast cell or a plant cell or a plant. The instant invention further pertains said transgenic cell or organism having an increased biosynthetic pathway for the production of substrates for the synthesis of triacylglycerol. A transgenic cell or organism having increased oil content is also contemplated within the scope of this invention.

- 20 Further, the invention pertains a transgenic cell or organism wherein the activity of an OEP encoded by one of the nucleic acid sequences SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 is increased in said cell or organism. The increased activity of OEP is characterized by an alteration in gene expression, catalytic activity and/or regulation of activity of the enzyme. Moreover, a transgenic cell or organism is included in the instant invention, wherein the increased biosynthetic pathway for the production of substrates for the production of triacylglycerol is characterized e.g. by the prevention of accumulation of undesirable fatty acids in the membrane lipids.

- 25 In a different embodiment, this invention also relates to methods of using a DNA sequence SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 for increasing the oil-content within the cells of different organisms.

- 30 Further, the invention makes possible a process for elevating the production of triacylglycerol, which comprises growing transgenic cells or organisms under conditions whereby the nucleotide sequences SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 are expressed in order to produce a protein in these cells with the ability of enhancing the production of triacylglycerol.

Corresponding genes coding for OEP can be isolated from other organisms, especially yeast-type organisms, like e.g. *Schizosaccharomyces pombe*, *Yarrowia lipolytica*.

*Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae*, *Emericella nidulans* and *Debaryomyces hansenii*.

Furthermore the invention pertains transgenic organisms comprising, in their genome or on a plasmid, a nucleic acid sequence SEQ ID NO :1, SEQ ID NO : 3 or SEQ ID NO : 5 according to the above, transferred by recombinant DNA technology. One important type of transgenic organism covered by this invention are commercially relevant plants in which said nucleotide sequence preferably would be expressed under the control of a storage organ specific promoter. Alternatively, the nucleotide sequence could also be expressed under the control of a seed-specific promoter or any other promoter suitable for tissue-specific high-level expression in plants.

The invention also pertains a protein encoded by a DNA molecule according to SEQ ID NO : 1, SEQ ID NO: 3 or SEQ ID NO: 5 or a functional biologically active fragment thereof having OEP activity in transgenic organisms. Alternatively, the invention pertains a protein produced in an organism, which has the amino acid sequence set forth in SEQ ID NO : 2, SEQ ID NO: 4 or SEQ ID NO: 6 or an amino acid sequence with at least 60% homology to said amino acid sequence having OEP activity.

The protein can be isolated from *Saccharomyces cerevisiae* or *Arabidopsis thaliana* but also from other organisms.

The invention additionally pertains the use of a protein according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6 or derivatives of that protein having OEP activity for the increased production of triacylglycerols, monoacylglycerol and/or fatty acids.

Surprisingly, it has been found that the homologous expression of the corresponding genes of *Arabidopsis thaliana* SEQ ID NO: 3 or SEQ ID NO: 5 in *Arabidopsis* leads to a significantly increased triacylglyceride (storage oils) content in the seeds as described in example 6. The oil content was increased by approximately 5%, in one transgenic line even by 10%, compared with wild-type control plants. The over-expression of the At140.1 or At140.2 genes had no adverse effects on the growth or other properties of the transformed plants.

Figure 1 shows the effect of deleting the YPR140w gene in yeast on in vitro lipid biosynthesis. The incorporation rate of [<sup>14</sup>C]glycerol-3phosphate ([<sup>14</sup>C]G3P) into lipids was analyzed in microsomal fractions of YPR140w-deletion and wt yeast (*Saccharomyces cerevisiae*) strains. Yeast strains were grown in rich media to an optical density of 1.5 (A<sub>600</sub>) and microsomal fractions were prepared. Enzymatic assays (n=4) were performed for 10 minutes at 30°C as described in Example 1.

The effect of over expressing gene YPR140w in yeast on the in vitro incorporation rate of [ $^{14}$ C]G3P into lipids is demonstrated in figure 2. The analyses were performed with crude extracts of *Saccharomyces cerevisiae* transformed with pAN3, multicopy plasmid with the gene YPR140w behind the GAL1 promotor, or pYES2, empty plasmid control as described in Example 2. Data presented are average from 8 replicates.

In figure 3 the total lipid content in the YPR140w yeast transformant harvested 24 hours (Exp.#1; open bar) or 28 hours (Exp.#2; filled bar) after induction is shown.

Yeast cells were transformed with the pAN3 vector expressing the YPR140w from the GAL1 inducible promotor. Yeast cells transformed with empty vector (pYES2) were used as control. Total lipid content was determined as nmol fatty acid (FA) per mg dry cell weight.

In figure 4 the total lipid content in the different yeast transformants harvested after 24 hours is shown. Yeast cells were transformed with the pY-At140.1 or the pY-At140.2 vector expressing the At140.1 or At140.2 from the GAL1 inducible promotor. Yeast cells transformed with empty vector (pYES2) were used as control. Total lipid content was determined as nmol fatty acid (FA) per mg dry cell weight.

In figure 5 the total lipid content of dry T2 generation seeds from *Arabidopsis* plants overexpressing At140.1 and At140.2 is shown. Wildtype *Arabidopsis* plants were used as control. Total lipid content was determined by conventional gas chromatography as mg FA per mg dry seeds.

The method according to the invention can be applied in principle to all plant species, in addition to the species *Arabidopsis thaliana*, which is employed as model plant. The method according to the invention is preferably applied to oil crops whose oil content is already naturally high and/or for the industrial production of oils.

Plant organism or tissue, organ, part, cell or propagation material thereof is generally understood as meaning any single- or multi-celled organism or a cell, tissue, part or propagation material (such as seeds or fruit) of same which is capable of photosynthesis. Included for the purpose of the invention are all genera and species of higher and lower plants of the Plant Kingdom. Annual, perennial, monocotyledonous and dicotyledonous plants are preferred. Also included are mature plants, seeds, shoots and seedlings, and parts, propagation material (for example tubers, seeds or fruits) and cultures derived from them, for example cell cultures or callus cultures.

## 10

Plant encompasses all annual and perennial monocotyledonous or dicotyledonous plants and includes by way of example, but not by limitation, those of the genera Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solarium, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesis, Pelargonium, Panieum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Pisum, Phaseolus, Lolium, Oryza, Zea, Avena, Hordeum, Secale, Triticum, Sorghum, Picea and Populus.

Preferred plants are those from the following plant families: Amaranthaceae, Asteraceae, Brassicaceae, Carophyllaceae, Chenopodiaceae, Compositae, Cruciferae, Cucurbitaceae, Labiatae, Leguminosae, Papilionoideae, Liliaceae, Linaceae, Malvaceae, Rosaceae, Rubiaceae, Saxifragaceae, Scrophulariaceae, Solanaceae, Sterculiaceae, Tetragoniaceae, Theaceae, Umbelliferae.

Preferred monocotyledonous plants are selected in particular from the monocotyledonous crop plants such as, for example, the Gramineae family, such as rice, maize, wheat or other cereal species such as barley, millet and sorghum, rye, triticale or oats, and sugar cane, and all grass species.

The invention is applied very particularly preferably to dicotyledonous plant organisms. Preferred dicotyledonous plants are selected in particular from the dicotyledonous crop plants such as, for example,

- Asteraceae such as Heliantus annuus (sunflower), tagetes or calendula and others,
- Compositae, especially the genus Lactuca, very particularly the species sativa (lettuce) and others,
- Cruciferae, particularly the genus Brassica, very particularly the species napus (oilseed rape), campestris (beet), oleracea cv Tastie (cabbage), oleracea cv Snowball Y (cauliflower) and oleracea cv Emperor (broccoli) and other cabbages; and the genus Arabidopsis, very particularly the species thaliana, and cress or canola and others,
- Cucurbitaceae such as melon, pumpkin/squash or zucchini and others.

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- Leguminosae, particularly the genus *Glycine*, very particularly the species *max* (soybean), soya, and alfalfa, pea, beans or peanut and others,
- 5 - Rubiaceae, preferably the subclass Lamiidae such as, for example *Coffea arabica* or *Coffea liberica* (coffee bush) and others,
- 10 - Solanaceae, particularly the genus *Lycopersicon*, very particularly the species *esculentum* (tomato), the genus *Solanum*, very particularly the species *tuberosum* (potato) and *melongena* (aubergine) and the genus *Capsicum*, very particularly the genus *annuum* (pepper) and tobacco or paprika and others,
- Sterculiaceae, preferably the subclass Dilleniidae such as, for example, *Theobroma cacao* (cacao bush) and others,
- 15 - Theaceae, preferably the subclass Dilleniidae such as, for example, *Camellia sinensis* or *Thea sinensis* (tea shrub) and others,
- Umbelliferae, particularly the genus *Daucus* (very particularly the species *carota* (carrot)) and *Apium* (very particularly the species *graveolens dulce* (celery)) and others;
- 20

and linseed, cotton, hemp, flax, cucumber, spinach, carrot, sugar beet and the various tree, nut and grapevine species, in particular banana and kiwi fruit.

- 25 Also encompassed are ornamental plants, useful or ornamental trees, flowers, cut flowers, shrubs or turf plants which may be mentioned by way of example but not by limitation are angiosperms, bryophytes such as, for example, Hepaticae (liverworts) and Musci (mosses); pteridophytes such as ferns, horsetail and clubmosses; gymnosperms such as conifers, cycades, ginkgo and Gnetatae; algae such as Chlorophyceae, Phaeophyceae, Rhodophyceae, Myxophyceae, Xanthophyceae, Bacillariophyceae (diatoms) and Euglenophyceae. Plants within the scope of the invention comprise by way of example and not by way of limitation, the families of the Rosaceae such as rose, Ericaceae such as rhododendron and azalea, Euphorbiaceae such as poinsettias and croton, Caryophyllaceae such as pinks, Solanaceae such as petunias, Gesneriaceae such as African violet, Balsaminaceae such as touch-me-not, Orchidaceae such as orchids, Iridaceae such as gladioli, iris, freesia and crocus, Compositae such as marigold, Geraniaceae such as geranium, Liliaceae such as dracena, Moraceae such as ficus, Araceae such as cheeseplant and many others.
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Furthermore, plant organisms for the purposes of the invention are further organisms capable of being photosynthetically active such as, for example, algae, cyanobacteria and mosses. Preferred algae are green algae such as, for example, algae from the genus *Haematococcus*, *Phaedactylum tricornatum*, *Volvox* or *Dunaliella*. *Synechocystis* is particularly preferred.

Most preferred are oil crops. Oil crops are understood as being plants whose oil content is already naturally high and/or which can be used for the industrial production of oils. These plants can have a high oil content and/or else a particular fatty acid composition which is of interest industrially. Preferred plants are those with a lipid content of at least 1% by weight. Oil crops encompassed by way of example: *Borvago officinalis* (borage); *Brassica* species such as *B. campestris*, *B. napus*, *B. rapa* (mustard, oilseed rape or turnip rape); *Cannabis sativa* (hemp); *Carthamus tinctorius* (safflower); *Cocos nucifera* (coconut); *Crambe abyssinica* (crambe); *Cuphea* species (Cuphea species yield fatty acids of medium chain length, in particular for industrial applications); *Elaeis guinensis* (African oil palm); *Elaeis oleifera* (American oil palm); *Glycine max* (soybean); *Gossypium hirsutum* (American cotton); *Gossypium barbadense* (Egyptian cotton); *Gossypium herbaceum* (Asian cotton); *Helianthus annuus* (sunflower); *Linum usitatissimum* (linseed or flax); *Oenothera biennis* (evening primrose); *Olea europaea* (olive); *Oryza sativa* (rice); *Ricinus communis* (castor); *Sesamum indicum* (sesame); *Triticum* species (wheat); *Zea mays* (maize), and various nut species such as, for example, walnut or almond.

"Total oil content" refers to the sum of all oils, preferably to the sum of the triacylglycerides.

"Oils" encompasses neutral and/or polar lipids and mixtures of these. Those mentioned in Table 1 may be mentioned by way of example, but not by limitation.

Neutrale lipids	Triacylglycerol (TAG)
	Diacylglycerol (DAG)
	Monoacylglycerol (MAG)
Polar lipids	Monogalactosyldiacylglycerol (MGDG)
	Digalactosyldiacylglycerol (DGDG)
	Phosphatidylglycerol (PG)
	Phosphatidylcholine (PC)
	Phosphatidylethanolamine (PE)
	Phosphatidylinositol (PI)
	Phosphatidylserine (PS)
	Sulfoquinovosyldiacylglycerol

Table 1: Classes of plant lipids

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Neutral lipids preferably refers to triacylglycerides. Both neutral and polar lipids may comprise a wide range of various fatty acids. The fatty acids mentioned in Table 2 may be mentioned by way of example, but not by limitation.

Nomenclature <sup>1</sup>	Name
16:0	Palmitic acid
16:1	Palmitoleic acid
16:3	Roughanic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	Linolenic acid
$\gamma$ -18:3-18:3	Gamma-linolenic acid *
20:0	Arachidic acid
22:6	Docosahexaenoic acid (DHA) *
20:2	Eicosadienoic acid
20:4	Arachidonic acid (AA) *
20:5	Eicosapentaenoic acid (EPA) *
22:1	Erucic acid

Table 2: Overview over various fatty acids (selection)

<sup>1</sup> Chain length: number of double bonds

\* not naturally occurring in plants

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Oils preferably relates to seed oils.

"Increase in" the total oil content refers to the increased oil content in a plant or a part, tissue or organ thereof, preferably in the seed organs of the plants. In this context, the oil content is at least 5%, preferably at least 10%, particularly preferably at least 15%, very particularly preferably at least 20%, most preferably at least 25% increased under otherwise identical conditions in comparison with a starting plant which has not been subjected to the method according to the invention, but is otherwise unmodified.

Conditions in this context means all of the conditions which are relevant for germination, culture or growth of the plant, such as soil conditions, climatic conditions, light conditions, fertilization, irrigation, plant protection treatment and the like.

"YPR140w" generally refers to all those proteins which are capable of increasing the oil content in oil producing organisms, especially microorganisms, yeast, fungi and plants and are identical to SEQ ID NO: 2 or have homology to SEQ ID NO: 2.

Yeast refers to the group of unicellular fungi with a pronounced cell wall and formation of pseudomycelium (in contrast to molds). They reproduce vegetatively by budding and/or fission (Schizosaccharomyces and Saccharomyces, respectively).

Encompassed are what are known as false yeasts, preferably the families Cryptococcaceae, Sporobolomycetaceae with the genera Cryptococcus, Torulopsis, Pityrosporum, Brettanomyces, Candida, Klöckera, Trigonopsis, Trichosporon, Rhodotorula and Sporobolomyces and Bullera, and true yeasts (yeasts which also reproduce sexually; ascus), preferably the families Endo- and Saccharomycetaceae, with the genera Saccharomyces, Debaryomyces, Lipomyces, Hansenula, Endomycopsis, Pichia, Hanseniaspora. Most preferred are the genera Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Zygosaccharomyces rouxii, und Yarrowia lipolytica, Emericella nidulans, Aspergillus nidulans, Debaryomyces hansenii and Torulaspora hansenii.

YPR140w refers in particular to the polypeptide sequence SEQ ID NO : 2.

Most preferably, YPR140w refers to the yeast protein OEP as shown in SEQ ID NO : 2 and functional equivalents or else functionally equivalent portions of the above.

"At140.1" and "At140.2" refer in particular to the polypeptide sequence SEQ ID NO : 4 and SEQ ID NO : 6.



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Most preferably, At140.1 and At140.2 refer to the *Arabidopsis thaliana* protein OEP as shown in SEQ ID NO : 4 and SEQ ID NO : 6 and functional equivalents or else functionally equivalent portions of the above.

- 5 Functional equivalents refers in particular to natural or artificial mutations of the yeast protein OEP as shown in SEQ ID NO : 2 and homologous polypeptides from other yeasts which have the same essential characteristics of YPR140w as defined above. Mutations encompass substitutions, additions, deletions, inversions or insertions of one or more amino acid residues.

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The YPR140w to be employed advantageously within the scope of the present invention can be found readily by database searches or by screening gene or cDNA libraries using the polypeptide sequence shown in SEQ ID NO : 2, which is given by way of example, or the nucleic acid sequence as shown in SEQ ID NO : 1, which

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encodes the latter, as search sequence or probe.

Said functional equivalents preferably have at least 60%, particularly preferably at least 70%, particularly preferably at least 80%, most preferably at least 90% homology with the protein of SEQ ID NO: 2.

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Functional equivalents refer in particular to natural or artificial mutations of the *Arabidopsis thaliana* OEP as shown in SEQ ID NO: 4 or SEQ ID NO : 6 and homologous polypeptides from other plants which have the same essential characteristics of an OEP as defined above. Mutations encompass substitutions, additions, deletions,

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inversions or insertions of one or more amino acid residues.

The nucleic acid to be employed advantageously within the scope of the present invention can be found readily by database searches or by screening gene or cDNA libraries using the polypeptide sequences shown in SEQ ID NO : 4 or SEQ ID NO : 6,

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which is given by way of example, or the nucleic acid sequence as shown in SEQ ID NO : 3 and SEQ ID NO : 5, which encode the latter, as search sequence or probe.

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Furthermore the following nucleic acid sequences can be used in order to identify and clone genes encoding an OEP from plant organisms and having at least 60% homology to SEQ ID NO : 3 or SEQ ID NO : 5 :

*Arabidopsis thaliana* ESTs from GenBank: AV782272, AV821735, AV825509, AI996105, AV791433, BE528444

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Glycine max ESTs from GenBank: AW458589, BQ740950, BI893252, BG154651, AW507932

Rose Petals EST from GenBank: BQ105746

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Rice EST from GenBank: AU166240

Tomato ESTs from GenBank: BG123776, AW218643

10 Medicago truncatula ESTs from GenBank: BG644974, CA858351

Gossypium arboreum EST from GenBank: BQ403497

Potato EST from GenBank: BE342183

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Furthermore the nucleic acid sequences SEQ ID NO : 7 to SEQ ID NO : 35 can be used in order to identify and clone genes encoding an OEP from plant organisms having at least 60 % homology to SEQ ID NO : 3 or SEQ ID NO : 5 .

20 Said functional equivalents have at least 60%, preferably at least 70%, particularly preferably at least 80%, most preferably at least 90% homology with the protein of SEQ ID NO : 4 or SEQ ID NO : 6.

25 The functional equivalent of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 has an identity of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57% preferably at least 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, and 70% more preferably 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85% most preferably at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity with the SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6.

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Homology between two polypeptides is understood as meaning the identity of the amino acid sequence over the entire sequence length which is calculated by comparison with the aid of the program algorithm GAP (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA), setting the following parameters:

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Gap Weight: 8

Length Weight: 2

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Average Match: 2,912

Average Mismatch: -2,003

For example, a sequence with at least 80% homology with the sequence SEQ ID NO: 2 at the protein level is understood as meaning a sequence which, upon comparison with the sequence SEQ ID NO: 2 with the above program algorithm and the above parameter set has at least 80% homology.

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Functional equivalents - for example - also encompass those proteins which are encoded by nucleic acid sequences which have at least 60%, particularly preferably at least 70%, particularly preferably at least 80%, most preferably at least 90% homology with the nucleic acid sequence with the SEQ ID NO: 1.

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Homology between two nucleic acid sequences is understood as meaning the identity of the two nucleic acid sequences over the entire sequence length which is calculated by comparison with the aid of the program algorithm GAP (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA),

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Gap Weight: 50

Length Weight: 3

Average Match: 10

Average Mismatch: 0

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For example, a sequence which has at least 80% homology with the sequence SEQ ID NO: 1 at the nucleic acid level is understood as meaning a sequence which, upon comparison with the sequence SEQ ID NO: 1 within the above program algorithm with the above parameter set has a homology of at least 80%.

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Functional equivalents also encompass those proteins which are encoded by nucleic acid sequences which hybridize under standard conditions with a nucleic acid sequence described by SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5, the nucleic acid sequence which is complementary thereto or parts of the above and which have the essential characteristics of an OEP as characterised by SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

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Natural examples of OEPs and the corresponding genes can furthermore readily be found in various organisms, in particular plants, whose genomic sequence is unknown, by hybridization techniques in a manner known per se, for example starting from the nucleic acid sequences SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

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The hybridization may be carried out under moderate (low stringency) or, preferably, under stringent (high stringency) conditions.

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Such hybridization conditions are described, inter alia, in Sambrook, J., Fritsch, E.F., Maniatis, T., in: Molecular Cloning (A Laboratory Manual), 2nd edition, Cold Spring Harbor Laboratory Press, 1989, pages 9.31-9.57 or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

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By way of example, the conditions during the washing step may be selected from the range of conditions which is limited by those with low stringency (with 2X SSC at 50°C) and those with high stringency (with 0.2X SSC at 50°C, preferably at 65°C) (20X SSC: 0.3 M sodium citrate, 3 M sodium chloride, pH 7.0).

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In addition, the temperature may be raised during the washing step from moderate conditions at room temperature, 22°C, to stringent conditions at 65°C.

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Both parameters, salt concentration and temperature, may be varied simultaneously and it is also possible to keep one of the two parameters constant and to vary only the other one. It is also possible to use denaturing agents such as, for example, formamide or SDS during hybridization. In the presence of 50% formamide, the hybridization is preferably carried out at 42°C.

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Some exemplary conditions for hybridization and washing step are listed below:

(1.) hybridization conditions with, for example

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(i) 4X SSC at 65°C, or

(ii) 6X SSC at 45°C, or

(iii) 6X SSC at 68°C, 100 mg/ml denatured fish sperm DNA, or

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(iv) 6X SSC, 0.5% SDS, 100 mg/ml denatured fragmented salmon sperm DNA at 68°C, or

(v) 6X SSC, 0.5% SDS, 100 mg/ml denatured fragmented salmon sperm DNA, 50% formamide at 42°C, or

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(vi) 50% formamide, 4X SSC at 42°C, or

(vii) 50% (vol/vol) formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer pH 6.5, 750 mM NaCl, 75 mM sodium citrate at 42°C, or

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- (viii) 2X or 4X SSC at 50°C (moderate conditions), or
- (ix) 30 to 40% formamide, 2X or 4X SSC at 42°C (moderate conditions).

5 (2.) Washing steps of 10 minutes each with, for example

- (i) 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or
- (ii) 0.1X SSC at 65°C, or
- 10 (iii) 0.1X SSC, 0.5% SDS at 68°C, or
- (iv) 0.1X SSC, 0.5% SDS, 50% formamide at 42°C, or
- 15 (v) 0.2X SSC, 0.1% SDS at 42°C, or
- (vi) 2X SSC at 65°C (moderate conditions).

20 The invention furthermore relates to transgenic expression constructs which can ensure a transgenic expression of an OEP as characterised by SEQ ID NO : 2, SEQ ID NO: 4 or SEQ ID NO: 6 in a plant organism or a tissue, organ, part, cells or propagation material of said plant organism.

25 The definition given above applies to an OEP, with the transgenic expression of an OEP described by the sequence with the SEQ ID NO: 4 or SEQ ID NO: 6 being particularly preferred.

30 In said transgenic expression constructs, a nucleic acid molecule encoding an OEP is preferably in operable linkage with at least one genetic control element (for example a promoter) which ensures expression in a plant organism or a tissue, organ, part, cell or propagation material of same.

Especially preferred are transgenic expression cassettes wherein the nucleic acid sequence encoding an OEP is described by

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- a) a sequence SEQ ID NO : 1, SEQ ID NO: 3 or SEQ ID NO: 5
- b) a sequence derived from a sequence SEQ ID NO : 1, SEQ ID NO: 3 or SEQ ID NO: 5 in accordance with the degeneracy of the genetic code

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- c) a sequence which has at least 60% identity with a sequence SEQ ID NO : 1, SEQ ID NO: 3 or SEQ ID NO: 5.

- Operable linkage is understood as meaning, for example, the sequential arrangement of a promoter with the nucleic acid sequence encoding an OEP which is to be expressed (for example the sequence as shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 and, if appropriate, further regulatory elements such as, for example, a terminator in such a way that each of the regulatory elements can fulfil its function when the nucleic acid sequence is expressed recombinantly. Direct linkage in the chemical sense is not necessarily required for this purpose. Genetic control sequences such as, for example, enhancer sequences can also exert their function on the target sequence from positions which are further removed or indeed from other DNA molecules. Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned behind the sequence acting as promoter so that the two sequences are linked covalently to each other. The distance between the promoter sequence and the nucleic acid sequence to be expressed recombinantly is preferably less than 200 base pairs, particularly preferably less than 100 base pairs, very particularly preferably less than 50 base pairs.
- Operable linkage and a transgenic expression cassette can both be effected by means of conventional recombination and cloning techniques as they are described, for example, in Maniatis T, Fritsch EF and Sambrook J (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY), in Silhavy TJ, Berman ML und Enquist LW (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY), in Ausubel FM et al. (1987) Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience and in Gelvin et al. (1990) In: Plant Molecular Biology Manual. However, further sequences which, for example, act as a linker with specific cleavage sites for restriction enzymes, or of a signal peptide, may also be positioned between the two sequences. Also, the insertion of sequences may lead to the expression of fusion proteins. Preferably, the expression cassette composed of a promoter linked to a nucleic acid sequence to be expressed can be in a vector-integrated form and can be inserted into a plant genome, for example by transformation.
- However, a transgenic expression cassette is also understood as meaning those constructs where the nucleic acid sequence encoding an OEP is placed behind an endogenous plant promoter in such a way that the latter brings about the expression of the OEP.

Promoters which are preferably introduced into the transgenic expression cassettes are those which are operable in a plant organism or a tissue, organ, part, cell or propagation material of same. Promoters which are operable in plant organisms is understood as meaning any promoter which is capable of governing the expression of genes, in particular foreign genes, in plants or plant parts, plant cells, plant tissues or plant cultures. In this context, expression may be, for example, constitutive, inducible or development-dependent.

The following are preferred:

a) Constitutive promoters

"Constitutive" promoters refers to those promoters which ensure expression in a large number of, preferably all, tissues over a substantial period of plant development, preferably at all times during plant development (Benfey et al. (1989) EMBO J 8:2195-2202). A plant promoter or promoter originating from a plant virus is especially preferably used. The promoter of the CaMV (cauliflower mosaic virus) 35S transcript (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. (1986) Plant Mol Biol 6:221-228) or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202) are especially preferred. Another suitable constitutive promoter is the Rubisco small subunit (SSU) promoter (US 4,962,028), the leguminB promoter (GenBank Acc. No. X03677), the promoter of the nopaline synthase from Agrobacterium, the TR dual promoter, the OCS (octopine synthase) promoter from Agrobacterium, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the SmaS promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the vacuolar ATPase subunits, the promoter of the Arabidopsis thaliana nitrilase-1 gene (GenBank Acc. No.: U38846, nucleotides 3862 to 5325 or else 5342) or the promoter of a proline-rich protein from wheat (WO 91/13991), and further promoters of genes whose constitutive expression in plants is known to the skilled worker. The CaMV 35S promoter and the Arabidopsis thaliana nitrilase-1 promoter are particularly preferred.

b) Tissue-specific promoters

Furthermore preferred are promoters with specificities for seeds, such as, for example, the phaseolin promoter (US 5,504,200; Bustos MM et al. (1989) Plant

Cell 1(9):839-53), the promoter of the 2S albumin gene (Joseffson LG et al. (1987) J Biol Chem 262:12196- 12201), the legumine promoter (Shirsat A et al. (1989) Mol Gen Genet 215(2):326-331), the USP (unknown seed protein) promoter (Bäumlein H et al. (1991) Mol Gen Genet 225(3):459-67), the napin gene promoter (US 5,608,152; Stalberg K et al. (1996) L Planta 199:515-519), the pro-moter of the sucrose binding proteins (WO 00/26388) or the legumin B4 promoter (LeB4; Bäumlein H et al. (1991) Mol Gen Genet 225: 121-128; Bäumlein et al. (1992) Plant Journal 2(2):233-9; Fiedler U et al. (1995) Biotechnology (NY) 13(10):1090f), the Arabidopsis oleosin promoter (WO 98/45461), and the Brassica Bce4 promoter (WO 91/13980).

Further suitable seed-specific promoters are those of the gene encoding high-molecular weight glutenin (HMWG), gliadin, branching enzyme, ADP glucose pyrophosphatase (AGPase) or starch synthase. Promoters which are furthermore preferred are those which permit a seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. The promoter of the lpt2 or lpt1 gene (WO 95/15389, WO 95/23230) or the promoters described in WO 99/16890 (promoters of the hordein gene, the glutelin gene, the oryzin gene, the prolamin gene, the gliadin gene, the glutelin gene, the zein gene, the casirin gene or the secalin gene) can advantageously be employed.

c) Chemically inducible promoters

The expression cassettes may also contain a chemically inducible promoter (review article: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108), by means of which the expression of the exogenous gene in the plant can be controlled at a particular point in time. Such promoters such as, for example, the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), a salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic acid-inducible promoter EP 0 335 528) or an ethanol-cyclohexanone-inducible promoter (WO 93/21334) can likewise be used. Also suitable is the promoter of the glutathione-S transferase isoform II gene (GST-II-27), which can be activated by exogenously applied safeners such as, for example, N,N-diallyl-2,2-dichloroacetamide (WO 93/01294) and which is operable in a large number of tissues of both monocots and dicots.

Particularly preferred are constitutive promoters, very particularly preferred seed-specific promoters, in particular the napin promoter and the USP promoter.



In addition, further promoters which make possible expression in further plant tissues or in other organisms such as, for example, *E. coli* bacteria, may be linked operably with the nucleic acid sequence to be expressed. Suitable plant promoters are, in principle, all of the above-described promoters.

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The nucleic acid sequences present in the transgenic expression cassettes according to the invention or transgenic vectors can be linked operably with further genetic control sequences besides a promoter. The term genetic control sequences is to be understood in the broad sense and refers to all those sequences which have an effect on the establishment or the function of the expression cassette according to the invention. Genetic control sequences modify, for example, transcription and translation in prokaryotic or eukaryotic organisms. The transgenic expression cassettes according to the invention preferably encompass a plant-specific promoter 5'-upstream of the nucleic acid sequence to be expressed recombinantly in each case and, as additional genetic control sequence, a terminator sequence 3'-downstream, and, if appropriate, further customary regulatory elements, in each case linked operably with the nucleic acid sequence to be expressed recombinantly.

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Genetic control sequences also encompass further promoters, promoter elements or minimal promoters capable of modifying the expression-controlling properties. Thus, genetic control sequences can, for example, bring about tissue-specific expression which is additionally dependent on certain stress factors. Such elements are, for example, described for water stress, abscisic acid (Lam E and Chua NH, *J Biol Chem* 1991; 266(26): 17131 -17135) and thermal stress (Schoffl F et al. (1989) *Mol Gen Genetics* 217(2-3):246-53).

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Further advantageous control sequences are, for example, in the Gram-positive promoters *amy* and *SPO2*, and in the yeast or fungal promoters *ADC1*, *MFa*, *AC*, *P-60*, *CYC1*, *GAPDH*, *TEF*, *rp28*, *ADH*.

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In principle all natural promoters with their regulatory sequences like those mentioned above may be used for the method according to the invention. In addition, synthetic promoters may also be used advantageously.

Genetic control sequences further also encompass the 5'-untranslated regions, introns or nonencoding 3'-region of genes, such as, for example, the actin-1 intron, or the *Adh1-S* intron 1, 2 and 6 (for general reference, see: *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)). It has been demonstrated that these may play a significant role in regulating gene expression. Thus, it has been demonstrated that 5'-untranslated sequences can enhance the transient expression of

heterologous genes. Translation enhancers which may be mentioned by way of example are the tobacco mosaic virus 5' leader sequence (Gallie et al. (1987) Nucl Acids Res 15:8693-8711) and the like. They may furthermore promote tissue specificity (Rouster J et al. (1998) Plant J 15:435-440).

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The transient expression cassette can advantageously contain one or more of what are known as enhancer sequences in operable linkage with the promoter, and these make possible an increased recombinant expression of the nucleic acid sequence. Additional advantageous sequences such as further regulatory elements or terminators may also be inserted at the 3' end of the nucleic acid sequences to be expressed recombinantly. One or more copies of the nucleic acid sequences to be expressed recombinantly may be present in the gene construct.

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Polyadenylation signals which are suitable as control sequences are plant polyadenylation signals, preferably those which correspond essentially to *Agrobacterium tumefaciens* T-DNA polyadenylation signals, in particular those of gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTIACHS (Gielen et al. (1984) EMBO J 3:835 et seq.) or functional equivalents thereof. Examples of particularly suitable terminator sequences are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

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Control sequences are furthermore understood as those which make possible homologous recombination or insertion into the genome of a host organism, or removal from the genome. In the case of homologous recombination, for example, the coding sequence of the specific endogenous gene can be exchanged in a directed fashion for a sequence encoding a dsRNA. Methods such as the cre/lox technology permit the tissue-specific, possibly inducible, removal of the expression cassette from the genome of the host organism (Sauer B (1998) Methods. 14(4):381-92). Here, certain flanking sequences are added to the target gene (lox sequences), and these make possible removal by means of cre recombinase at a later point in time.

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A recombinant expression cassette and the recombinant vectors derived from it may comprise further functional elements. The term functional element is to be understood in the broad sense and refers to all those elements which have an effect on generation, replication or function of the expression cassettes, vectors or transgenic organisms according to the invention. Examples which may be mentioned, but not by way of limitation, are:

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- a) Selection markers which confer resistance to a metabolism inhibitor such as 2-deoxyglucose-6-phosphate (WO 98/45456), antibiotics or biocides, preferably

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- herbicides, such as, for example, kanamycin, G 418, bleomycin, hygromycin, or phosphothricin and the like. Particularly preferred selection markers are those which confer resistance to herbicides. The following may be mentioned by way of example: DNA sequences which encode phosphinothricin acetyltransferases (PAT) and which inactivate glutamine synthase inhibitors (bar and pat gene), 5-enolpyruvylshikimate-3-phosphate synthase genes (EPSP synthase genes), which confer resistance to Glyphosate (N-(phosphonomethyl)glycine), the gox gene, which encodes Glyphosate-degrading enzyme (Glyphosate oxidoreductase), the deh gene (encoding a dehalogenase which inactivates dalapon), sulfonylurea- and imidazolinone-inactivating acetolactate synthases, and bxn genes which encode nitrilase enzymes which degrade bromoxynil, the aasa gene, which confers resistance to the antibiotic spectinomycin, the streptomycin phosphotransferase (SPT) gene, which permits resistance to streptomycin, the neomycin phosphotransferase (NPTII) gene, which confers resistance to kanamycin or geneticidin; the hygromycin phosphotransferase (HPT) gene, which confers resistance to hygromycin, the acetolactate synthase gene (ALS), which confers resistance to sulfonylurea herbicides (for example mutated ALS variants with, for example, the S4 and/or Hra mutation).
- b) Reporter genes which encode readily quantifiable proteins and which allow the transformation efficacy or the expression site or time to be assessed via their color or enzyme activity. Very particularly preferred in this context are reporter proteins (Schenborn E, Groskreutz D. Mol Biotechnol. 1999; 13(1):29-44) such as the "green fluorescent protein" (GFP) (Sheen et al.(1995) Plant Journal 8(5):777-784), chloramphenicol transferase, a luciferase (Ow et al. (1986) Science 234:856-859), the aequorin gene (Prasher et al. (1985) Biochem Biophys Res Commun 126(3):1259-1268),  $\beta$ -galactosidase, with  $\beta$ -glucuronidase being very particularly preferred (Jefferson et al. (1987) EMBO J 6:3901-3907).
- c) Replication origins which allow replication of the expression cassettes or vectors according to the invention in, for example, E.coli. Examples which may be mentioned are ORI (origin of DNA replication), the pBR322 ori or the P15A ori (Sambrook et al.: Molecular Cloning. A Laboratory Manual, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
- d) Elements which are required for agrobacterium-mediated plant transformation such as, for example, the right or left border of the T-DNA, or the vir region.
- To select cells which have successfully undergone homologous recombination or else cells which have successfully been transformed, it is generally required additionally to

introduce a selectable marker which confers resistance to a biocide (for example a herbicide), a metabolism inhibitor such as 2-deoxyglucose-6-phosphate (WO 98/45456) or an antibiotic to the cells which have successfully undergone recombination. The selection marker permits the selection of the transformed cells from untransformed cells (McCormick et al. (1986) Plant Cell Reports 5:81-84).

In addition, said recombinant expression cassette or vectors may comprise further nucleic acid sequences which do not encode a nucleic acid sequence SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 and whose recombinant expression leads to a further increase in fatty acid biosynthesis. By way of example, but not by limitation, such a proOIL nucleic acid sequence which is additionally expressed recombinantly can be selected from among nucleic acids encoding acetyl-CoA carboxylase (ACCase), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidate acyltransferase (LPAT), diacylglycerol acyltransferase (DAGAT) and phospholipid:diacylglycerol acyltransferase (PDAT). Such sequences are known to the skilled worker and are readily accessible from databases or suitable cDNA libraries of the respective plants.

An expression cassette according to the invention can advantageously be introduced into an organism or cells, tissues, organs, parts or seeds thereof (preferably into plants or plant cells, tissues, organs, parts or seeds) by using vectors in which the recombinant expression cassettes are present. The invention therefore furthermore relates to said recombinant vectors which encompass a recombinant expression cassette for a nucleic acid sequence SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

For example, vectors may be plasmids, cosmids, phages, viruses or else agrobacteria. The expression cassette can be introduced into the vector (preferably a plasmid vector) via a suitable restriction cleavage site. The resulting vector is first introduced into E.coli. Correctly transformed E.coli are selected, grown, and the recombinant vector is obtained with methods known to the skilled worker. Restriction analysis and sequencing may be used for verifying the cloning step. Preferred vectors are those which make possible stable integration of the expression cassette into the host genome.

The invention furthermore relates to transgenic plant organisms or tissues, organs, parts, cells or propagation material thereof which comprise a SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 as defined above, a transgenic expression cassette for a SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 or a transgenic vector encompassing such an expression cassette.

Such a transgenic plant organism is generated, for example, by means of transformation or transfection of the corresponding proteins or nucleic acids. The generation

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Cell Reports 5:81-84). The plants obtained can be bred and hybridized in the customary manner. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary.

- 5 The above-described methods are described, for example, in Jenes B et al. (1993) Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by SD Kung and R Wu, Academic Press, pp.128-143, and in Potrykus (1991) Annu Rev Plant Physiol Plant Molec Biol 42:205-225). The construct to be expressed is preferably cloned into a vector which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al. (1984) Nucl Acids Res 12:8711f).
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- Once a transformed plant cell has been generated, an intact plant can be obtained using methods known to the skilled worker. For example, callus cultures are used as starting material. The development of shoot and root can be induced in this as yet undifferentiated cell biomass in the known fashion. The plantlets obtained can be planted out and used for breeding.
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- The skilled worker is familiar with such methods for regenerating plant parts and intact plants from plant cells. Methods which can be used for this purpose are, for example, those described by Fennell et al. (1992) Plant Cell Rep. 11: 567-570; Stoeger et al (1995) Plant Cell Rep. 14:273-278; Jahne et al. (1994) Theor Appl Genet 89:525-533.
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- "Transgenic", for example in the case of an OEP, refers to a nucleic acid sequence, an expression cassette or a vector comprising said OEP nucleic acid sequence or to an organism transformed with said nucleic acid sequence, expression cassette or vector or all those constructs established by recombinant methods in which either
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- a) the nucleic acid sequence encoding an OEP or
- 30 b) a genetic control sequence, for example a promoter which is functional in plant organisms, which is linked operably with said nucleic acid sequence under a)

- are not in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to be, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment refers to the natural chromosomal locus in the source organism or the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably retained, at least to some extent. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, particularly preferably
- 35
- 40

at least 1000 bp, very particularly preferably at least 5000 bp. A naturally occurring expression cassette, for example the naturally occurring combination of the promoter of a gene coding for an OEP with the corresponding OEP gene, becomes a transgenic expression cassette when the latter is modified by non-natural, synthetic ("artificial") methods such as, for example, a mutagenization. Such methods are described in US 5,565,350; WO 00/15815; see also above.

Host or starting organisms which are preferred as transgenic organisms are, above all, plants in accordance with the above definition. Included for the purposes of the invention are all genera and species of higher and lower plants of the Plant Kingdom, in particular plants which are used for obtaining oils, such as, for example, oilseed rape, sunflower, sesame, safflower, olive tree, soya, maize, wheat and nut species. Furthermore included are the mature plants, seed, shoots and seedlings, and parts, propagation material and cultures, for example cell cultures, derived therefrom. Mature plants refers to plants at any desired developmental stage beyond the seedling stage. Seedling refers to a young, immature plant at an early developmental stage.

The transgenic organisms can be generated with the above-described methods for the transformation or transfection of organisms.

The invention furthermore relates to the use of the transgenic organisms according to the invention and to the cells, cell cultures, parts - such as, for example, in the case of transgenic plant organisms roots, leaves and the like - and transgenic propagation material such as seeds or fruits which are derived therefrom for the production of food-stuffs or feedstuffs, pharmaceuticals or fine chemicals, in particular oils, fats, fatty acids or derivatives of these.

Besides influencing the oil content, the transgenic expression of SEQ ID NO : 1, SEQ ID NO : 3, SEQ ID NO : 5 or derivatives thereof in plants may mediate yet further advantageous effects such as, for example, an increased stress resistance. Such osmotic stress occurs for example in saline soils and water and is an increasing problem in agriculture. Increased stress tolerance makes it possible, for example, to use areas in which conventional arable plants are not capable of thriving for agricultural usage.

The invention now having been generally described will be more readily understood by reference to the following examples, which are included for the purpose of illustration only, and are not intended to limit scope of the present invention.



## Examples

## General methods:

- 5 Unless otherwise specified, all chemicals were from Fluka (Buchs), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Restriction enzymes, DNA-modifying enzymes and molecular biological kits were from Amersham-Pharmacia (Freiburg), Biometra (Göttingen), Roche (Mannheim), New England Biolabs (Schwalbach), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt),
- 10 Qiagen (Hilden), Stratagen (Amsterdam, Netherlands), Invitrogen (Karlsruhe) and Ambion (Cambridgeshire, United Kingdom). The reagents used were employed in accordance with the manufacturer's instructions.

- 15 For example, oligonucleotides can be synthesized chemically in the known manner using the phosphoramidite method (Voet, Voet, 2<sup>nd</sup> edition, Wiley Press New York, pages 896-897). The cloning steps carried out for the purposes of the present invention such as, for example, restriction cleavages, agarose gel electrophoreses, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of *E. coli* cells, bacterial cultures, multiplication
- 20 of phages and sequence analysis of recombinant DNA, are carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6. Recombinant DNA molecules were sequenced using an ABI laser fluorescence DNA sequencer following the method of Sanger (Sanger et al. (1977) Proc Natl Acad Sci USA 74:5463-5467).

25

## Example 1

Decreased incorporation of [<sup>14</sup>C]Glycerol 3-Phosphate into lipids of a yeast strain in which the *YPR140w* gene was disrupted.

30

- A haploid yeast (*Saccharomyces cerevisiae*) strain, (EUROSCARF accession no. Y15555; EUROSCARF, University Frankfurt, D-60439 Frankfurt, Germany,) with the *YPR140w* gene deleted, and the corresponding wild type (wt) strain By4742 (*MAT $\alpha$  his3 $\Delta$ 1, leu2  $\Delta$ 0, lys2  $\Delta$ 0, ura3  $\Delta$ 0*), were acquired from the EUROSCARF yeast
- 35 strain collection. Yeast cells were grown in liquid YPD (1% yeast extract, 2% peptone and 2% glucose). For preparing microsomal membranes two yeast strains, Y15555 and wt, were grown in 750 ml cultures in liquid YPD on rotary shaking at 30° C. The cultures were started by inoculation from overnight liquid-YPD pre-cultures to an OD<sub>600</sub> of 0.05 and the cells were harvested at an OD<sub>600</sub> of about 1.5, by centrifugation for five
- 40 minutes at 3000rpm. The cell pellets were washed once in deionised water and frozen.

Cell pellets were thawed and washed with breaking buffer (0.6 M Mannitol, 20mM Tris-HCl pH 7.6, 1mM EDTA, 1µg/ml Aprotinin, 0.7µg/ml Pepstatin, 0.5 µg/ml Leupeptin) before being centrifuged for five minutes at 30000 rpm. The cells were then re-suspended in breaking buffer (one ml of breaking buffer per g of wet cell pellet) and then broken by passing them 2-3 times through a French Press (Ammco SLM instruments, Urbana Illinois). The cells were diluted 1.5 times with breaking buffer and centrifuged at 1000g for five minutes at 4°C. The supernatant was then centrifuged two additional times, first at 5000g for 10 minutes then at 100,000 g for sixty minutes both at 4°C. The microsomal pellet was re-suspended in 20mM Tris-HCl, pH 7.6 to a protein concentration of approximately 10mg/ml and homogenized with glass homogenizer. Microsomes were stored frozen at -70°C. Protein concentrations were determined using the BCA method (Pierce) with BSA as standard protein. The enzymatic incorporation of [<sup>14</sup>C]glycerol-3-phosphate were analysed by adding 50µl of an reaction mixture (50mM Tris-HCl pH 7.5, 8 mM NaF, 4mM MgCl<sub>2</sub>, 0.4 mM 10000dpm/nmol [<sup>14</sup>C]-G3P, 4 mg/ml BSA, 0.2mM 16:0-CoA, 2 mM DTT) to 5µl of microsomal preparations in a final volume of 100 µl. After incubation at 30°C for 10 min the reactions were stopped by adding 400µl of methanol/chloroform/ glacial acetic acid (50:50:1) and 100µl of water followed by thoroughly shaking and centrifugation for twenty seconds at 13000 rpm. The bottom chloroform layer was removed and transferred into scintillation vials containing 4 ml of scintillation fluid (ethanol/ethanol 2:1, 0.4% 2(1-butylphenol)-5-(4-biph-enyl)-1, 3, 4-oxadizole). Radioactivity counting was carried out in a LKB Wallace 1290 Rackbeta Liquid scintillation counter.

The effect of deleting the *YPR140w* gene in yeast was studied on the *in vitro* biosynthesis of lipids by analysing the incorporation rate of [<sup>14</sup>C]glycerol-3phosphate into lipids. The glycerol-3-phosphate acyltransferase (GPAT) reaction is the first step in the lipid biosynthesis in yeast, both for phospholipids and triacylglycerols. The lysophosphatidic acid formed by GPAT activity can be further metabolised into phosphatidic acid and diacylglycerol by other enzymes which are part of the so called Kennedy pathway which are also present in the microsomal membrane fraction. The method used in this experiment to analyse the incorporation of [<sup>14</sup>C]glycerol-3phosphate into lipids, is taking advantage of the fact that the labelled substrate is water soluble while the products are lipid soluble. This means that a simple chloroform extraction with scintillation counting of the chloroform phase will give the amount of incorporated [<sup>14</sup>C]glycerol-3phosphate into lipids, which is the same as the GPAT activity as all labelled lipid products formed has to pass the GPAT acylation step. The *YPR140w*-deletion and wild type yeast strains were grown in liquid cultures and microsomal membrane fractions were prepared from harvested cell pellets. Removing the gene had little or no effect on the growth rate as determined by optical density measurements. However, the incorporation of [<sup>14</sup>C]glycerol-3phosphate into lipids when microsomal membranes

were incubated in the GPAT assay was clearly reduced to about 60% of wild type, see figure 1. These results indicate that the *YPR140w* gene might encode a GPAT enzyme or another type of protein that affects microsomal GPAT activity indirectly.

## 5 Example 2

Increased incorporation of [ $^{14}$ C]Glycerol 3-Phosphate into lipids in crude extracts of wt yeast over expressing the gene *YPR140w*.

- 10 For induced high-level expression of the *YPR140w* gene, a 1146 bp DNA fragment as described in SEQ ID NO: 1, from start to stop codon, was amplified from *S.cerevisiae* W303 genomic DNA by using a 1:1 mixture of *Taq* and *pfu* DNA polymerases with the 5' primer, ATGTCTTTTAGGGATGTCCTAGA, and the 3' primer, TCAATCATCCTTACCCTTTGGTT. The resulting PCR product was gel purified,
- 15 incubated with *Taq* polymerase and TA-cloned into the pCR2.1-TOPO cloning vector (Invitrogen). The *YPR140w* gene was then excised from the cloning vector by *EcoRI* digestion and cloned into the *EcoRI* behind the strong inducible *GAL1* promoter in the multicopy plasmid pYES2 (Invitrogen), thus generating the plasmid pAN3. The wt yeast strain By4742 (*MAT $\alpha$  his3 $\Delta$ 1, leu2  $\Delta$ 0, lys2  $\Delta$ 0, ura3  $\Delta$ 0*), was transformed with pAN3
- 20 and pYES2 using standard protocol. Overnight precultures of the two yeast transformants, wt yeast containing pAN3 or pYES2, were grown in 15 ml of synthetic liquid media lacking uracil and supplemented with 2 % Glucose (vol/vol). All yeast cultures were grown on rotary shaking at 30°C. The precultures were used to inoculate sixty ml of synthetic liquid media lacking uracil and supplemented with 3% glycerol and 3%
- 25 lactate to a start OD<sub>600</sub> of 0.2. The sixty ml cultures were grown for forty-eight hours (OD<sub>600</sub> about 1.5) and then the *GAL1* over expression promoter was induced by the addition of galactose to a final concentration of 2% (vol/vol) and the cultures were shaken for a further 6 hours at 30°C. Cells were harvested (OD<sub>600</sub> about 2.5) by centrifugation five minutes at 3000rpm. Pellets were washed once with water and
- 30 frozen. Cell pellets were thawed, resuspended in ice-cold breaking buffer (20mM Tris-HCl pH 7.6, 1mM EDTA, 1µg/ml Aprotinin, 0.7µg/ml Pepstatin, 0.5 µg/ml Leupeptin) to 1,5 ml and added to two ml screw cap tubes containing 1 ml of acid washed glass beads (diameter 0.45-0.5 mm). The tubes were shaken at 4°C for 3 minutes in a Minibeadbeater-8 (Techtum lab and then centrifuged for 10 minutes at 3000 rpm. The
- 35 supernatant was harvested and stored at -70°C. Protein concentrations were determined using the BCA-method (Pierce) with BSA as protein standard. Enzymatic assays were run for ten minutes at 30°C. The samples (100µg of protein) were diluted to 50 µl with water and the assay was started by adding 50µl of assay mixture (50mM Tris-HCl pH 7.5, 8 mM NaF, 4mM MgCl<sub>2</sub>, 0.4 mM 10000dpm/nmol [ $^{14}$ C]-G-3-P, 4 mg/ml BSA,
- 40 0.2mM 16:0-CoA, 2 mM DTT). The assays were stopped by adding 400 µl of methan-

ol/chloroform/ glacial acetic acid (50:50:1) and 100µl of water followed by thoroughly shaking and centrifugation for twenty seconds at 13000 rpm. The bottom chloroform layer was removed and transferred into scintillation vials containing 4 ml of scintillation fluid (ethanol/ethanol 2:1, 0.4% 2(1-butylphenol)-5-(4-biphenyl)-1, 3, 4-oxa-dizole).

- 5 Radioactivity counting was carried out in a LKB Wallace 1290 Rackbeta Liquid scintillation counter.

10 The effect of over expression of the *YPR140w* gene was studied on the rate of *in vitro* incorporation of [ $^{14}$ C]glycerol-3phosphate into lipids by crude cellular extracts. Over expression of the *YPR140w* gene was achieved by cloning the gene behind the strong galactose inducible *GAL1* promotor and transforming the generated plasmid, pAN3, into wt yeast. The over expression plasmid, pYES2, without any gene behind the *GAL1* promoter was used as an "empty plasmid" control in the experiment. Incorporation of glycerol-3phosphate into lipids is catalyzed by the GPAT (glycerol 3-phosphate acyltransferase) enzyme, which utilizes acyl-CoA as acyldonor for the reaction and gives lysophosphatidic acid as product. The GPAT reaction is the first step in the lipid biosynthesis in yeast, both for phospholipids and triacylglycerols. The formed lyso-phosphatidic acid can be further metabolized into phosphatidic acid and diacylglycerol by other enzymes, which are also present in the crude cellular extracts and are part of the so-called Kennedy pathway. The method used in this experiment to analyze the GPAT activity, or the incorporation of [ $^{14}$ C]glycerol-3phosphate into lipids, is taking advantage of the fact that the labeled substrate is water-soluble while the products are lipid soluble, and that all labeled lipid products formed have to pass the GPAT step. This means that a simple chloroform extraction with scintillation counting of the chloroform phase will give the amount of incorporated [ $^{14}$ C]glycerol-3phosphate into lipids, which is the same as the GPAT activity.

30 Induced high-level over expression of the *YPR140* gene SEQ ID NO: 1 from the pAN3 plasmid in wt yeast increased the incorporation of [ $^{14}$ C]glycerol-3phosphate into lipids 2.5 fold as compared to empty plasmid control, see figure 2. The increase in incorporation of [ $^{14}$ C]glycerol-3phosphate into lipids is an effect of increased GPAT activity, but this does not mean that the *YPR140w* gene product necessarily has to be a GPAT. The *in vitro* assay is done in a crude extract containing a very complex mixture of enzymes and proteins. Over expression of *YPR140w* SEQ ID NO: 1 in wt yeast leads to an increased GPAT activity.

## Example 3

Triacylglycerol accumulation in yeast cells expressing the *YPR140w* gene

- 5 For induced high-level expression of the *YPR140w* gene, a 1146 bp DNA fragment as described in SEQ ID NO: 1, from start to stop codons, was amplified from *S. cerevisiae* W303 genomic DNA by using a 1:1 mixture of *Taq* and *pfu* DNA polymerases with the 5' primer, ATGTCTTTTAGGGATGTCCTAGA, and the 3' primer, TCAATCATCCTTACCCTTTGGTT. The resulting PCR product was gel purified,  
10 incubated with *Taq* polymerase and TA-cloned into the pCR2.1-TOPO cloning vector (Invitrogen). The *YPR140w* gene was then excised from the cloning vector by *EcoRI* digestion and cloned into the *EcoRI* behind the strong inducible GAL1 promoter in the multicopy plasmid pYES2 (Invitrogen), thus generating the plasmid pAN3. The wild type yeast strain By4742 (*MAT $\alpha$  his3 $\Delta$ 1, leu2  $\Delta$ 0, lys2  $\Delta$ 0, ura3  $\Delta$ 0*), transformed with the pAN3 was cultivated at 30 °C on a rotary shaker in synthetic medium (Sherman, F.  
15 et al., (1986) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Lab. Press, Plainview, NY. ) lacking uracil and supplemented with 2 % (vol/vol) glycerol and 2 % (vol/vol) ethanol. The GAL1 promoter was induced after 24 hours of growth by the addition of 2 % (wt/vol) final concentration of galactose. Cells were  
20 harvested after an additional 24 or 28 hours of growth. Wild type cells By4742, transformed with the empty vector (pYES2) and cultivated under identical conditions, were used as a control.

- 25 In order to quantify the total lipid content, 3 x 5 ml aliquots from yeast cultures were harvested by centrifugation, and the resulting pellets were washed with distilled water and lyophilised. The weight of the dried cells was determined, and the fatty acid content was quantified by conventional gas-liquid chromatography (GLC) analyses after conversion to methyl esters (Dahlqvist et al. (2000) *Proc. Natl. Acad. Sci. USA* 97, 6487 – 6492).

- 30 The lipid content was then calculated as nmol fatty acids per mg dry weight. The lipid composition of the yeast was determined in cells harvested from 35-ml liquid cultures. The harvested yeast cells were re-suspended in 15 ml glass tubes in water to a final volume of 0.6 ml, to which 3.75 ml chloroform: methanol (1:2), 50  $\mu$ l acetic acid, and  
35 2 ml of glass beads (0.45 – 0.50 mm) were added. The yeast cells were disrupted by vigorous agitation (5 x 1 min) and the lipids were extracted into chloroform according to standard method (Bligh, E.G. and Dyer, W.J., *Can. J. Biochem. Physiol.* 37(1959), 911-917). The collected lipid fraction was divided in two parts and separated by TLC on Silica Gel 60 plates (Merck) in hexane / diethyl ether / acetic acid (70:30:1) for the  
40 quantification of neutral lipids, i.e. unesterified fatty acids (FA), diacylglycerols (DAG),

triacylglycerols (TAG), and steryl esters (SE), and in chloroform / methanol / acetic acid: water (85: 15: 10: 3.5) for the quantification of the major polar lipids, i.e. phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). The lipid areas were located by brief exposure to I<sub>2</sub> vapors and identified by means of appropriate standards. The different lipid classes were excised from the plates and fatty acid methyl esters were prepared by heating the excised material at 85°C for 60 min in 2% (vol/vol) sulfuric acid in dry methanol. The methyl esters were extracted and quantified by conventional gas-liquid chromatography (GLC) analyses as described in Dahlqvist et al., 2000.

The effect of high-level expression of the *YPR140w* gene SEQ ID NO: 1 on lipid accumulation was studied by transforming the wild-type yeast strain By4742 with the pAN3 plasmid containing the *YPR140w* gene, under control of the galactose-induced *GAL1* promoter. The high-level expression of the *YPR140w* gene from this promoter did not retard the growth rate as determined by optical density measurements. The expression of the *YPR140w* gene was induced after 24 h and cells were harvested after an additional 24 hours (Exp.#1) or 28 hours (Exp.#2) of cultivation. The total lipid content was determined as nmol fatty acids per mg dry weight and was shown to be significantly higher in cells expressing the *YPR140w* gene as compared to cells transformed with an empty vector. The total lipid content of the *YPR140w* (pAN3) transformants harvested 24 hours after induction (Exp.#1 in Fig. 3) was 16 %, as compared to control cells (pYES2). Similarly the lipid content was 12 % higher (Exp.#2 in Fig. 3) when harvested 28 hours after induction as compared to control cells. Furthermore, no major changes in the overall fatty acid composition occurred by the over expression of the *YPR140w* gene. The content of neutral lipid species was examined and this analysis clearly demonstrates that the major neutral lipid, triacylglycerol, is strongly elevated in cells over expressing the *YPR140w* (pAN3) as compared to control cells (table 3). The triacylglycerol content in the *YPR140w* (pAN3) transformant harvested 28 hours after induction (Exp.#2 in table 3) was 62 %, as compared to the empty vector control. In cells harvested 24 hours after induction (Exp.#1 in table 3) the triacylglycerol content was similarly elevated although less pronounced. It is therefore concluded that the triacylglycerol content is clearly increased in yeast cells by the over expression of the *YPR140w* gene SEQ ID NO: 1. The content of steryl esters is low in the control cells and was less affected by the over expression of this gene.

	Lipid content (nmol FA per mg dry weight)									
	Polar lipids		DAG		FA		TAG		SE	
	Exp#1	Exp#2	Exp#1	Exp#2	Exp#1	Exp#2	Exp#1	Exp#2	Exp#1	Exp#2
pYES2	81	99	7.2	7.8	6.6	6.0	92	104	4.9	5.6
pAN3	72	67	9.7	5.0	6.3	1.3	130	169	5.9	7.0

Table 3.

- 5 The lipid content in yeast transformants. Yeast cells, were transformed with the pAN3 vector expressing the *YPR140w* SEQ ID NO: 1 from the GAL1 inducible promotor, and cells trans-formed with empty vector (pYES2) were used as control. The content of diacylglycerol (DAG), unesterified fatty acids (FA), triacylglycerol (TAG), and steryl esters (SE) was determined in yeast cells harvested 24 hours after induction and
- 10 calculated as nmol fatty acid (FA) per mg dry cell weight.

- 15 A significant decrease in the overall polar lipid content was also observed due to the elevated expression of the *YPR140w* gene. The polar lipid content per dry cell weight decreased with up to 32% (table 4). Moreover, the relative distribution of the major polar lipid species as presented in table 1 differs between the overexpressor and the control cells. The relative content of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) is decreased whereas phosphatidylcholine (PC) and phosphatidylserine (PS) is increased in the *YPR140w* transformant.

	Relative content of polar lipids (%)			
	PI	PS	PC	PE
pYES2	10,6	10,3	47,0	32,1
pAN3	4,8	12,0	59,1	24,1

20

Table 4.

- 25 The relative composition (%) of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the different transformants harvested after 28 hours after induction. Yeast cells were transformed with the pAN3 vector expressing the *YPR140w* SEQ ID NO: 1 from the GAL1 inducible promoter. Yeast cells transformed with empty vector (pYES2) were used as control.

In summary, the triacylglycerol content in yeast cells expressing *YPR140w* SEQ ID NO: 1 from a strong promoter was increased with 61 % as compared to the control, which demonstrates the potential use of these genes in increasing the oil content in transgenic organisms.

5

#### Example 4

Identification of genes homologous to *YPR140w* in plants.

- 10 To identify possible homologues to the *YPR140w* yeast gene SEQ ID NO: 1, the NCBI database were searched for related plant sequences. From these BLAST searches two protein sequences in *Arabidopsis thaliana* were identified with 31,6 % (At140.1) and 26.3% (At140.2) sequence identity to the deduced amino acid sequence of the open reading frame *YPR140w* using the GAP programme. From an EST database the full-length sequences of the corresponding cDNAs were identified and named *At140.1* and
- 15 *At140.2*. The *Arabidopsis At140.1* and *At140.2* genes were then PCR amplified from an *Arabidopsis* seedling cDNA library. The *At140.1* gene was amplified from the cDNA by Pfu Turbo DNA polymerase (Stratagene, USA) with the use of the primers At140.1-S1: GTCGGTCTTTCTAACTGAATC and At140.1-A1: CCTGTGGGACTTAAACCTCA.
- 20 The PCR product was diluted 10 times and used as a template in a second PCR with internal primers At140.1-S2: CAGAATGGGAATTCATTTTG and At140.1-A2: CTAACGGGAGTTTAACTTGCA. Similarly, the *At140.2* gene was amplified from the cDNA by using the primers At140.2-S1: CTGGTCTCGTTTCTAATTG and At140.2-A1: CATGGCGAATCTAAACCGGAAC. The resulting DNA fragments were purified and
- 25 cloned into pCR-Blunt II-TOPO (Invitrogen life technologies, USA), generating pCR-*At140.1* and pCR-*At140.2*, respectively. The genes were verified by sequencing, see SEQ ID NO : 3 and SEQ ID NO : 5.

#### Example 5

30

Triacylglycerol accumulation in yeast cells expressing the plant genes *At140.1* or *At140.2*

- 35 The *At140.1* and the *At140.2* genes SEQ ID NO: 3 and SEQ ID NO: 5 were excised from the cloning vectors pCR-*At140.1* and pCR-*At140.2* by BamHI and NotI digestions, respectively. The excised genes were then subcloned into the BamHI and NotI sites, respectively, behind the strong inducible GAL1 promotor in the multicopy plasmid pYES2 (Invitrogen, USA), thus generating the plasmids pY-*At140.1* and pY-*At140.2*, respectively. The wild type yeast strain By4742 (*MAT $\alpha$  his3 $\Delta$ 1, leu2  $\Delta$ 0, lys2  $\Delta$ 0, ura3  $\Delta$ 0*), transformed with these plasmids were cultivated at 30 °C on a rotary shaker
- 40



in synthetic medium (Sherman et al., 1986) lacking uracil and supplemented with 2 % (vol/vol) glycerol and 2 % (vol/vol) ethanol. The *GAL1* promoter was induced after 24 hours of growth by the addition of 2 % (wt/vol) final concentration of galactose. Cells were harvested after an additional 24 hours of growth. Wild type cells By4742 transformed with the empty vector, pYES2 and cultivated under identical conditions were used as a control. The lipid content of the yeast cells was determined as described in Example 3.

The effect of high-level expression of the *At140.1* (SEQ ID NO: 3) or the *At140.2* gene (SEQ ID NO: 5) on lipid accumulation was studied in yeast transformants expressing the *At140.1* or the *At140.2* gene, respectively, under control of the galactose-induced *GAL1* promoter. The gene expression was induced after 24 hours and cells were harvested after an additional 24 hours of cultivation. High-level expression of these genes showed only minor effects on the growth rate as determined by optical density measurements. However, the total lipid content, determined as nmol fatty acids (FA) per mg yeast, was strongly elevated in the *At140.1* or the *At140.2* transformants as compared to cells transformed with an empty vector. The total lipid content (Fig. 4) of the *At140.1* (pY-*At140.1*) and *At140.2* (pY-*At140.2*) transformants was 42 % and 45 % higher, respectively, as compared to control cells (pYES2). The increased lipid content is mainly explained by the strongly elevated triacylglycerol content in the *At140.1* and the *At140.2* transformants (table 5). The triacylglycerol content in these transformants was 79 % and 92 % higher, respectively, as compared to the empty vector control. The polar lipid content was slightly lowered and contents of unesterified fatty acids and diacylglycerols were increased whereas the steryl ester content was not affected by the over expression of the Arabidopsis genes, *At140.1* and *At140.2*.

	Lipid content (nmol FA per mg dry weight)				
	Polar lipids	DAG	FA	TAG	SE
pYES2	83	5.0	3.6	73	6.7
PY- <i>At140.1</i>	75	9.1	21	131	7.3
PY- <i>At140.2</i>	70	8.8	24	140	6.8

Table 5.

The lipid content in yeast transformants. Yeast cells, were transformed with the pY-*At140.1* or the pY-140.2 vector expressing the *At140.1* or the *At140.2* from the *GAL1* inducible promoter. Yeast cells transformed with empty vector (pYES2) were used as control. The content of diacylglycerol (DAG), unesterified fatty acids (FA), triacyl-

glycerol (TAG), and steryl esters (SE) was determined in yeast cells harvested 24 hours after induction and calculated as nmol fatty acid (FA) per mg dry cell weight.

5 The major polar lipids in yeast are phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The effects on the relative distribution of these major polar lipid classes by the over expression of the *At140.1* or the *At140.2* gene are presented in table 6. The most significant effect was observed in the phosphatidylinositol (PI) content of the *At140.1* or the *At140.2* transformants, with a 39% and a 33% decrease in the relative content as compared to  
10 control cells.

	Relative content of polar lipids (%)			
	PI	PS	PC	PE
<b>pYES2</b>	11.0	12.2	54.3	29.7
<b>pY-<i>At140.1</i></b>	6.7	11.1	48.7	33.4
<b>pY-<i>At140.2</i></b>	7.4	11.9	47.5	33.2

Table 6.

15 The relative composition (%) of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the different transformants harvested after 24 hours after induction. Yeast cells were transformed with the pY-*At140.1* or the pY-*At140.2* vector expressing the *At140.1* or the *At140.2* gene, respectively, from the GAL1 inducible promoter. Yeast cells transformed with empty  
20 vector (pYES2) were used as control.

25 The effect on the fatty acid profile by the over expression of the *At140.1* or the *At140.2* gene were also analysed. Only minor changes in response to the over expression of these genes on the fatty acid composition of the polar lipids were observed (data not shown). However, the levels of oleic (18:1) acid increased and the palmitic (16:0) decreased in triacylglycerols accumulated in yeast cells expressing the *At140.1* or the *At140.2* gene (table 7). A minor increase in the stearic acid (18:0) content was also observed.

	Relative fatty acid composition in triacylglycerol (%)			
	16:0	16:1	18:0	18:1
pYES2	30.5	31.5	13.2	24.8
pY-At140.1	14.5	31.3	15.4	38,3
pY-At140.2	14.2	31.4	15.0	39.3

Table 7.

5 The fatty acid composition of triacylglycerols in yeast cells transformed with the pY-At140.1 or the pY-At140.2 vector expressing the *At140.1* or the *At140.2* gene, respectively, from the GAL1 inducible promoter. Yeast cells transformed with empty vector (pYES2) were used as control. Major fatty acids present in yeast are palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acid.

10 In summary, the triacylglycerol content in yeast cells expressing the *At140.1* (SEQ ID NO: 3) or the *At140.2* gene (SEQ ID NO: 5) from a strong promoter was increased with 78 or 92 % as compared to the control, which demonstrates the potential use of these genes in increasing the oil content in transgenic organisms.

#### 15 Example 6

Transgenic plants expressing the *At140.1* or *At140.2* gene

20 *Cloning and analysis of oil content in plant:* For induced high-level expression of the *At140.1* (SEQ ID NO: 3) or *At140.2* gene (SEQ ID NO: 5) in plants, the genes were cloned into a binary vector system under the control of either a seed-specific or a constitutive promoter. An *Agrobacterium*-mediated plant transformation binary vector system was applied, including the primary cloning vector pART7-35S with a CaMV35S promoter or pART7-Nap with the seed specific napin promoter from *Brassica napus* (Josefsson, L. G. et al., J. Biol. Chem. 262 (1987), 12196-12201) coupled with a transcriptional terminator sequence and a binary Ti vector pGII0229 (Hellens, R.P. et al., Plant Mol. Biol. 42 (2000), 819-832). A DNA fragment, containing gene *At140.1*, was excised by BamHI and EcoRV from the cloning vector pCR-At140.1 (described in example 4) and was then subcloned into BamHI and SmaI treated pART7-35S or pART7-Nap, generating p35S-At140.1 and pNap-At140.1, respectively. From these plasmids the expression cassettes containing the promoter, the *At140.1* gene and the transcriptional terminator sequence was excised by NotI cleavage. The fragments were then inserted into the NotI site of the pGII0229 plasmid, generating the vectors pGII-35S-At140.1 and pGII-Nap-At140.1, respectively.

Plant expression vectors with the gene At140.2 expressed from a constitutive CaMV35S or from a seed specific napin promoter were constructed. First the NotI site was first deleted from the pCR-At140.2 by cutting with NotI, filling and re-ligation.

5 Thereafter the At140.2 gene was released by HindIII and XbaI and inserted into the corresponding sites of the plasmids pART7-35S or pART7-Nap, generating plasmids p35S-At140.2 and pNap-At140.2, respectively. The expression cassettes were then excised from these plasmids and introduced into NotI site of pGII0229, generating plasmids pGII-35S-At140.2 and pGII-Nap-At140.2, respectively.

10 Using floral dip essentially as described by Clough and Bent, 1998, plants were transformed with *Agrobacterium tumefaciens* GV3101 harboring the plasmid pGII-35S-At140.1, pGII-Nap-At140.1, pGII-35S-At140.2 and pGII-Nap-At140.2 respectively. Entire plants (inflorescence and rosette) were submerged for 20 – 30 sec in the infiltration media consisting of 5% sucrose, 0.02% Silwet L-77 (Osi Specialties, Danbury, CT) and re-suspended transformed *A. tumefaciens* cells. Plants were then transferred to a growth chamber with a photoperiod of 16 h of light at 21°C and 8 h of dark at 18°C (70% humidity). The T1 seeds were collected from mature plants. Subsequently transformed plants were identified on selection media by growing T1 seeds on MS-agar plates supplemented with BASTA (gulfosinate ammonium) 15 mg/l, cefotaxime 50 mg/L. Alternatively T1 plants were grown on soil trays and sprayed three times at an interval of two days with 0.4% BASTA.

25 Plant lipids can be extracted from plant material as described by Cahoon et al. (1999, Proc. Natl. Acad. Sci. USA 96, 22:12935-12940) and Browse et al. (1986, Anal. Biochemistry 442:141-145). Qualitative and quantitative lipid or fatty acid analysis is described in Christie, William W., Advances in Lipid Methodology. Ayr/Scotland :Oily Press. - (Oily Press Lipid Library; Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland :Oily Press, 1989 Repr. 1992. - IX,307).

30 The oil content of T2 seeds of the *Arabidopsis* transformants was analyzed by the use of conventional gas-liquid chromatography (GLC) and calculated as mg fatty acids per mg dry seeds (Fig. 5). As control, seeds from wild type plants were used. The over expression of the At140.1 (SEQ ID NO: 3) or the At140.2 gene (SEQ ID NO: 5) does result in an increase in total lipid content.

We claim:

1. A method of increasing the total oil content in a plant organism or a tissue, organ, part, cell or propagation material thereof, comprising
  - a) the transgenic expression of a polypeptide SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 in said plant organism or in a tissue, organ, part, cell or propagation material thereof, and
  - b) the selection of plant organisms in which - in contrast to or comparison with the starting organism - the total oil content in said plant organism or in a tissue, organ, part, cell or propagation material thereof is increased.
2. The method as claimed in claim 1, wherein the oil biosynthesis enhancing protein is encoded by a nucleic acid sequence selected from the group consisting of:
  - a) a nucleic acid sequence comprising a nucleotide sequence which is at least 60% identical to the nucleic acid sequence of SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5;
  - b) a nucleic acid sequence comprising a fragment of at least 30 nucleotides of a nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5;
  - c) a nucleic acid sequence which encodes a polypeptide comprising an amino acid sequence at least about 60% identical to the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 and
  - d) a nucleic acid sequence which encodes a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 or wherein the fragment comprises at least 10 contiguous amino acid residues of the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6.
3. A method as claimed in claim 1 or 2, wherein the plant is an oil crop.
4. A method as claimed in claim 1 or 2, wherein the total oil content in the seed of a plant is increased.

5. An expression cassette comprising in combination with a regulatory sequence a nucleic acid sequence selected from the group consisting of:
- a) a nucleic acid sequence comprising a nucleotide sequence which is at least 60% identical to the nucleotide sequence of SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5,
  - b) a nucleic acid sequence comprising a fragment of at least 30 nucleotides of a nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO : 1, SEQ ID NO : 3 or SEQ ID NO : 5,
  - c) a nucleic acid sequence which encodes a polypeptide comprising an amino acid sequence at least about 60% identical to the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6, or
  - d) a nucleic acid sequence which encodes a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6 wherein the fragment comprises at least 10 contiguous amino acid residues of the amino acid sequence of SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6

wherein said regulatory sequence is capable of mediating expression of said nucleic acid sequence in a plant.

- 6. An expression cassette according to claim 5, wherein said nucleic acid sequence encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO : 2, SEQ ID NO : 4 or SEQ ID NO : 6.
- 7. An expression cassette as claimed in claim 5 or 6, wherein the promoter is a seed-specific promotor.
- 8. A genetically modified plant organism or tissue, organ, part, cell or propagation material thereof, comprising a polypeptide as defined in SEQ ID NO : 2, SEQ ID NO 4 or SEQ ID NO 6 or an expression cassette as claimed in any of claims 5 to 7.

9. A genetically modified plant organism as claimed in claim 8, wherein the plant organism is selected from the group of the oil crops consisting of *Borvago officinalis*, *Brassica campestris*, *Brassica napus*, *Brassica rapa*, *Cannabis sativa*, *Carthamus tinctorius*, *Cocos nucifera*, *Crambe abyssinica*, *Cuphea species*, *Elaeis guinensis*, *Elaeis oleifera*, *Glycine max*, *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium herbaceum*, *Helianthus annuus*, *Linum usitatissimum*, *Oenothera biennis*, *Olea europaea*, *Oryza sativa*, *Ricinus communis*, *Sesamum indicum*, *Triticum species*, *Zea mays*, walnut and almond.
- 5
10. The use of a genetically modified plant organism or tissue, organ, part, cell or propagation material thereof as claimed in claim 8 or 9 for the production of oils, fats, free fatty acids or derivatives of the above.
- 10

46

Use of genes for increasing the oil content in plants

Abstract

- 5 The invention relates to methods for increasing the oil content in plants, preferably in plant seeds, by expressing the Ypr140w polypeptide from yeast or corresponding polypeptides from plants. The invention furthermore relates to expression constructs for expressing the yeast polypeptide Ypr140w or corresponding polypeptides from plants in plants, preferably in plant seeds, the transgenic plants expressing the polypeptide
- 10 and to the use of said transgenic plants for the production of food, feed, seed, pharmaceuticals or fine chemicals, in particular for the production of oils.



FIG. 1

5

10

15

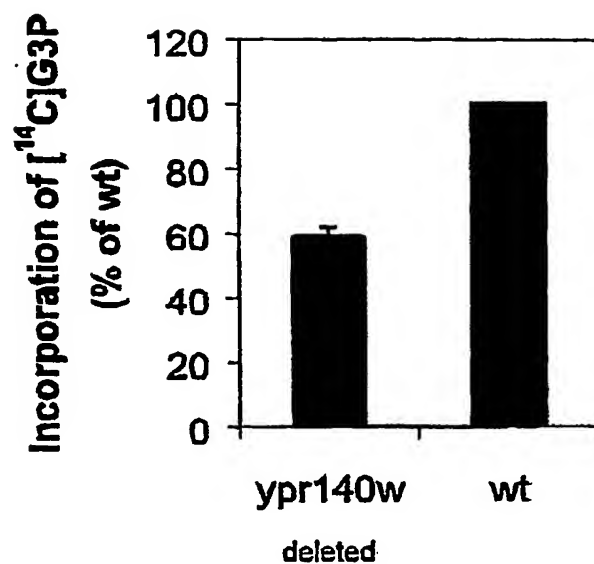


FIG. 2

5

10

15

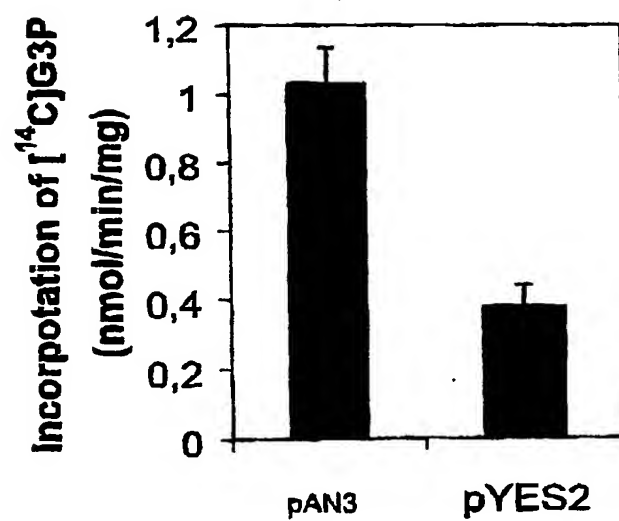


FIG. 3

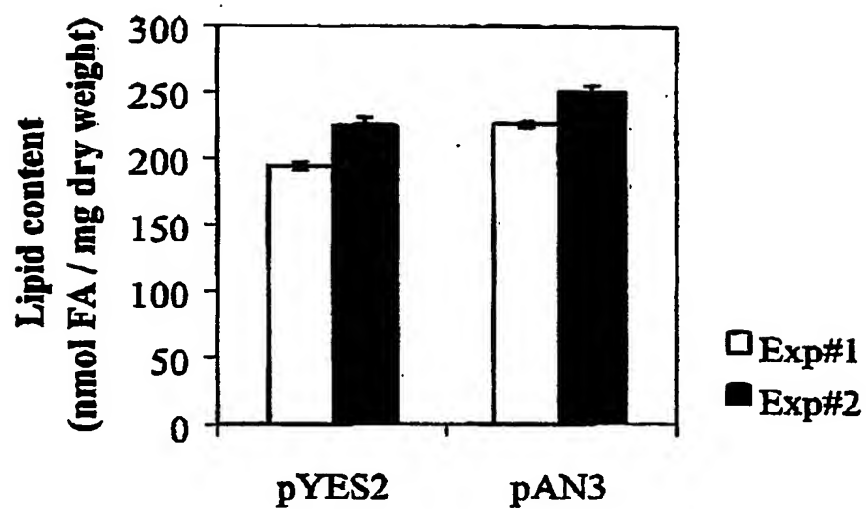


FIG. 4

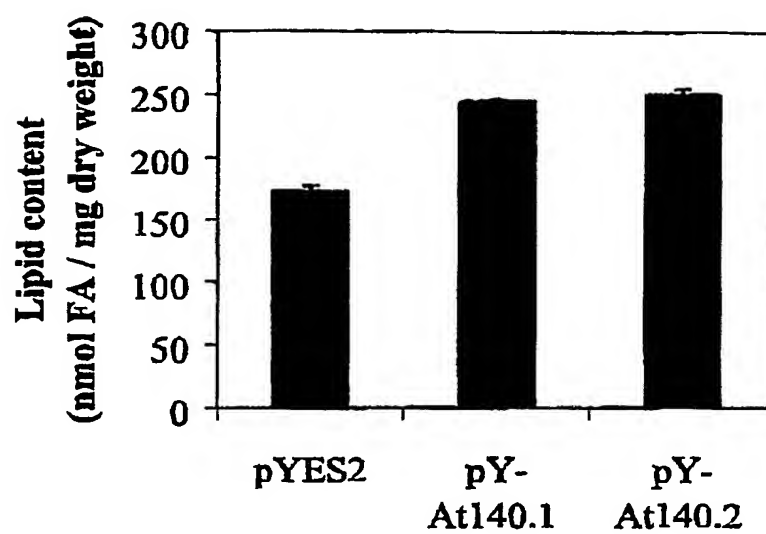
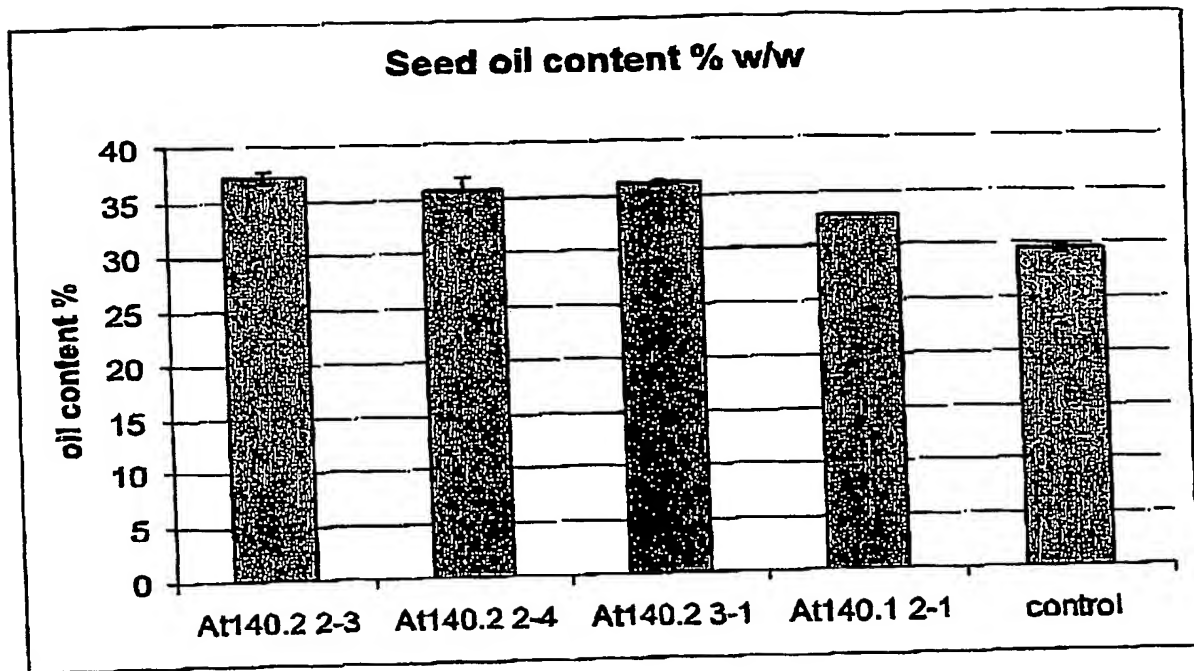


FIG. 5



BASF Plant Science GmbH

20020908

PF 54384 EP

1

SEQUENCE LISTING

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BASF Plant Science GmbH

20020908

PF 54384 EP

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	tgg gca gca aga cct gat cat ttg gga gga att cca agg aat act gtg Trp Ala Ala Arg Ser Asp His Leu Gly Gly Ile Pro Arg Asn Thr Val						103

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	10	15	20	
5	ata atg gct gtt agt gca ttt gca aaa gca gta gca aat ctt tgc aat Ile Met Ala Val Ser Ala Phe Ala Lys Ala Val Ala Asn Leu Cys Asn 25 30 35	151		
10	aaa agc tca gtt cac aat gca gat act ctt atg aat ctt gtc cag tca Lys Ser Ser Val His Asn Ala Asp Thr Leu Met Asn Leu Val Gln Ser 40 45 50	199		
15	aga cca cct ggt gtt cct ctc atc act gtt agt aat cac atg tgc act Arg Pro Pro Gly Val Pro Leu Ile Thr Val Ser Asn His Met Ser Thr 55 60 65 70	247		
20	ttg gat gat cca gta atg tgg ggg gca ttt aag ggt ctc ctt tcc tta Leu Asp Asp Pro Val Met Trp Gly Ala Phe Lys Gly Leu Leu Ser Leu 75 80 85	295		
25	gat cca gaa ttg gct cgg tgg gtt ctt gct gca gag gat ata tgt ttc Asp Pro Glu Leu Ala Arg Trp Val Leu Ala Ala Glu Asp Ile Cys Phe 90 95 100	343		
30	agg aac cct ata ttc tcc tac att ttc cgc act gga aaa tgt ata cct Arg Asn Pro Ile Phe Ser Tyr Ile Phe Arg Thr Gly Lys Cys Ile Pro 105 110 115	391		
35	ata act aga ggt ggt gga atc tac caa gaa aac atg aat gaa gct ctc Ile Thr Arg Gly Gly Gly Ile Tyr Gln Glu Asn Met Asn Glu Ala Leu 120 125 130	439		
40	cag cga tta aaa gat gga tct tgg ctg cat acc ttc cca gag gga aag Gln Arg Leu Lys Asp Gly Ser Trp Leu His Thr Phe Pro Glu Gly Lys 135 140 145 150	487		
45	gtg ttt caa gat gat gtt cct ata aga cga ctt aaa tgg gga act gca Val Phe Gln Asp Val Pro Ile Arg Arg Leu Lys Trp Gly Thr Ala 155 160 165	535		
50	agc ctc atc gcc cgt tcc cca gtt acc cca atc gtt ttg cca ata att Ser Leu Ile Ala Arg Ser Pro Val Thr Pro Ile Val Leu Pro Ile Ile 170 175 180	583		
55	cac cgt ggt ttt gag gag atg atg ccg gag aac tac aat aat gga cga His Arg Gly Phe Glu Glu Met Met Pro Glu Asn Tyr Asn Asn Gly Arg 185 190 195	631		
60	aga cca ctg gta ccg ttg ccg aac aaa cac ctt aaa gtt gtg gtt ggt Arg Pro Leu Val Pro Leu Pro Asn Lys His Leu Lys Val Val Val Gly 200 205 210	679		
65	gaa cca att gag ttt gat gtt cca atg atg gtt gag act gct gtc ttg Glu Pro Ile Glu Phe Asp Val Pro Met Met Val Glu Thr Ala Val Leu 215 220 225 230	727		
70	gac tcc cgc cat gta acc cct cct ctt caa gaa gtg aaa tgg cct gtc Asp Ser Arg His Val Thr Pro Pro Leu Gln Glu Val Lys Trp Pro Val 235 240 245	775		
75	ctc act tct gct ggc caa gtg cta gac gaa act gct cag aga cac ctc	823		

9

Leu Thr Ser Ala Gly Gln Val Leu Asp Glu Thr Ala Gln Arg His Leu  
 250 255 260

5    tac ata gct ctg tcc gag aag att caa tcc tcc ttg gaa aca ttg aga    871  
      Tyr Ile Ala Leu Ser Glu Lys Ile Gln Ser Ser Leu Glu Thr Leu Arg  
      265 270 275

10    ctc tta gcc aag cgg ttg tgacttccgc cgggaaactt catatcaaaa    919  
      Leu Leu Ala Lys Arg Leu  
      280

     tccggttcac aacttttaaa gttccgggtt agattcgcca tg    961

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      <212> PRT  
      <213> Arabidopsis thaliana

20    <400> 6  
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      1 5 10 15

25    Ile Pro Arg Asn Thr Val Ile Met Ala Val Ser Ala Phe Ala Lys Ala  
      20 25 30

     Val Ala Asn Leu Cys Asn Lys Ser Ser Val His Asn Ala Asp Thr Leu  
      35 40 45

30    Met Asn Leu Val Gln Ser Arg Pro Pro Gly Val Pro Leu Ile Thr Val  
      50 55 60

     Ser Asn His Met Ser Thr Leu Asp Asp Pro Val Met Trp Gly Ala Phe  
      65 70 75 80

35    Lys Gly Leu Leu Ser Leu Asp Pro Glu Leu Ala Arg Trp Val Leu Ala  
      85 90 95

40    Ala Glu Asp Ile Cys Phe Arg Asn Pro Ile Phe Ser Tyr Ile Phe Arg  
      100 105 110

     Thr Gly Lys Cys Ile Pro Ile Thr Arg Gly Gly Gly Ile Tyr Gln Glu  
      115 120 125

45    Asn Met Asn Glu Ala Leu Gln Arg Leu Lys Asp Gly Ser Trp Leu His  
      130 135 140

     Thr Phe Pro Glu Gly Lys Val Phe Gln Asp Asp Val Pro Ile Arg Arg  
      145 150 155 160

50    Leu Lys Trp Gly Thr Ala Ser Leu Ile Ala Arg Ser Pro Val Thr Pro  
      165 170 175

     Ile Val Leu Pro Ile Ile His Arg Gly Phe Glu Glu Met Met Pro Glu  
      180 185 190

55    Asn Tyr Asn Asn Gly Arg Arg Pro Leu Val Pro Leu Pro Asn Lys His  
      195 200 205

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Leu Lys Val Val Val Gly Glu Pro Ile Glu Phe Asp Val Pro Met Met  
 210 215 220

5 Val Glu Thr Ala Val Leu Asp Ser Arg His Val Thr Pro Pro Leu Gln  
 225 230 235 240

Glu Val Lys Trp Pro Val Leu Thr Ser Ala Gly Gln Val Leu Asp Glu  
 245 250 255

10 Thr Ala Gln Arg His Leu Tyr Ile Ala Leu Ser Glu Lys Ile Gln Ser  
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15

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<220>  
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 25 <222> (1)..(403)

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 gagagggggg gggatttatc aagaacacat gaatgaagcc cttgacgtgc tragaaatgg 240  
 aggttggtcg catacattcc ctgaaggaaa aatagcccaa gaagatcagc cgattagaag 300  
 attgaagtgg ggaactgccg gtcttatctg ccgagcacct ataactccaa tagttttgac 360  
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35

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 <211> 423  
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 40 <213> Glycine max

<220>  
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 <222> (1)..(423)

45

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 ttcattgect tcttcacat ctctctatcg taaacgagtg attaaggatt tcagttctga 180  
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 ctgtcacttg tttatgaacg gattaaacca ggtgcaggcg tatggtttag aaaaactgca 300  
 ctccgcgttg ctgcatagac ctaagggcaa acctcttctt acggtcagca atcatgttgc 360  
 tccatggat gatecgettg ttatcgcttc gctgcttctt tcgagtgttc tcttggatgc 420  
 tan 423

55

<210> 9  
 <211> 408  
 <212> DNA

11

&lt;213&gt; Glycine max

&lt;220&gt;

&lt;221&gt; misc\_feature

5 &lt;222&gt; (1)..(408)

&lt;220&gt;

&lt;221&gt; misc\_feature

10 &lt;222&gt; (1)..(408)

&lt;400&gt; 9

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 cgtcttctct caacaccgce gatgttcaca acggcgacac tctctccgc ctctccgct 180  
 ccagacccca ccgcgtcccc ctctattaccg ttagcaatca catgtccact ttggatgate 240  
 cgggttatgtg ggggttcaag ggttttctta tcttcgacac caacttagct cgtggggttc 300  
 tcgctgcccga agatatttgc ttcagggaatg cctctatcc cratattttt cggctgcata 360  
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20

&lt;210&gt; 10

&lt;211&gt; 368

&lt;212&gt; DNA

25 &lt;213&gt; Linum usitatissimum

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (1)..(368)

30 &lt;400&gt; 10

cggtagtag ccatgttget tctgtcgatg acccatttgt gattgcttca ttgctaccac 60  
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 taggaatccc gtcaacttctg cattcttaag actgtcaaag tcttgccctt ctctcggtgt 180  
 catggagttt atcagaagggt tatggacacg gcaattgcca acgtgaacac tgggtggctgg 240  
 gtacacatct tcccggaggt agccgttcta aggatggtgg gaaactatg ggggtctatca 300  
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 gtgcacac 368

40

&lt;210&gt; 11

&lt;211&gt; 376

&lt;212&gt; DNA

&lt;213&gt; Linum usitatissimum

45

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (1)..(376)

50 &lt;400&gt; 11

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 ctctacttctg cctagtctga tctcggccgc ctggtgtacc tctcatcact gttagcaacc 180  
 acatgtcaac gttagatgat cctctgargt ggggattcaa gggattccca atcatgggat 240  
 gcgaasttgt ttcgatgggt atgggctgct gaagacatct gtttcaggaa ttcttttcat 300  
 tottacctct ttcgcatggg gaaatgtatt cccattacaa gaggtggggg aatttatcgg 360  
 agccacatga atgaag 376

55

&lt;210&gt; 12



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12

<211> 418  
 <212> DNA  
 <213> *Linum usitatissimum*

5 <220>  
 <221> misc\_feature  
 <222> (1) .. (418)

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 gctgaagaca tctgtttcag gaattctttt cattcttact tctttcgcgt ggggaaatgt 180  
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 ttgagcaatg gtgattggct gcacacattc cctgagggaa aggtcaacca agaaattgga 300  
 15 cctataagac gattgaaatg gggaaactgcc agtctcatcg tccgtgcccc tgttacaccg 360  
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 20 <211> 445  
 <212> DNA  
 <213> *Glycine max*

<220>  
 25 <221> misc\_feature  
 <222> (1) .. (445)

<400> 13  
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 gactcgttgc cgtcgtccac cterttttat cgcaaacgag tgattaagga ttccagttct 180  
 gaagaagatt caactcttgc tcgtatgatg caagctgttg cggttcctgt tcttggaaat 240  
 gtctgtcagc tgtttatgaa cggattaaac agtgtgcagg tatatgggtt agaaaaactg 300  
 cactccgctt tactgcaaag acctaaagga aaacctcttc ttacggtcag caateatgtt 360  
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<210> 14  
 40 <211> 361  
 <212> DNA  
 <213> *Hordeum vulgare*

<220>  
 45 <221> misc\_feature  
 <222> (1) .. (361)

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 a 361

<210> 15  
 <211> 472

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&lt;212&gt; DNA

&lt;213&gt; Brassica napus

&lt;220&gt;

5 &lt;221&gt; misc\_feature

&lt;222&gt; (1)..(472)

&lt;400&gt; 15

10 tgtatcggaa tttccgggtc gacgaccacc gccggagagc cgcgggtttta tatacggacg 60  
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 ctctcccttc tgcccccgct ttttatcgca gaagagtacc taaagatttg acggcagaag 180  
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 gtcattgttt catgaatggc ctaaacgctg ttcagggtga tggcttagag aagttgcatg 300  
 atgctctgct caacaggcca aagaacaagc ctctcgtaac ggtagcaat catgtggcat 360  
 15 ccttggatga tccatttgtc attgcttcgt tacttccgcc taagcttcta ctgatgctc 420  
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&lt;210&gt; 16

20 &lt;211&gt; 412

&lt;212&gt; DNA

&lt;213&gt; Brassica napus

&lt;220&gt;

25 &lt;221&gt; misc\_feature

&lt;222&gt; (1)..(412)

&lt;400&gt; 16

30 ttttagatgat ccagtaatgt ggggaggggt caaggcgtct tctttcctta gatacagagc 60  
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 tcttccgcac tggcaagtgt atacctataa cttagaggtcg tggaatctac caagaacaca 180  
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 aggtgtttca agaagatgtg cctataagac gacttaaatg ggaaccgca agcctcatcg 300  
 cccgttgccc agtcacccca ategtcttgc caataattca ccgtggtttg acgagatgaa 360  
 35 tgcgagaggt acatttatgtg aaaangacca ccgtaccctg tgggaacaaa an 412

&lt;210&gt; 17

40 &lt;211&gt; 410

&lt;212&gt; DNA

&lt;213&gt; Brassica napus

&lt;220&gt;

45 &lt;221&gt; misc\_feature

&lt;222&gt; (1)..(410)

&lt;400&gt; 17

50 ttttagatgat ccagtaatgt ggggaggggt caagggtctt ctctccttag atccagagct 60  
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 gagtgaagct ctcgagcgat taaaagatgg atcttgggtg cataccttc cagagggcaa 240  
 ggtgtttcaa gaagatgtgc ctataagac acttaaatgg ggaaccgcaa gctcatcgc 300  
 ccgttgccca gtcacccaaa tcgtcttgc aatatttcac cgtggttttg acaacatgat 360  
 55 gccggaabaat gtccttttat ggaagaatga caaccgtacc tgtggggaan 410

&lt;210&gt; 18

&lt;211&gt; 420

&lt;212&gt; DNA

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&lt;213&gt; Glycine max

&lt;220&gt;

&lt;221&gt; misc\_feature

5 &lt;222&gt; (1) .. (420)

&lt;400&gt; 18

gcccattggcg cgcgcgacac aaccaccaca cctttatccc ctccgcogat ggctacttct 60  
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 10 cgtccacctc tttttatcgc atacgagtga ttaaggattt cagttctgaa gaagattcaa 180  
 ctcttggtcg tatgatgcaa gctggtgagg ttctgtttct tggaaatgtc tgtcacgtgt 240  
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 tgcaaaagacc caaaggaaaa cctcttctta cggtcagcaa tcatgttgct tccatggatg 360  
 15 atcctcttgt tattgcttcg ctgcttctcc cgagtggtct tttggaagct aggaatctcn 420

&lt;210&gt; 19

&lt;211&gt; 490

&lt;212&gt; DNA

20 &lt;213&gt; Brassica napus

&lt;220&gt;

&lt;221&gt; misc\_feature

25 &lt;222&gt; (1) .. (490)

&lt;400&gt; 19

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 tactgggtatg caagatataa tgcctatagg agccagtggt ccacggattg gcaaaacagt 180  
 30 gacagtgate attggagatc ctattccctt taatgacct gtagaractg aaggagccaa 240  
 acacgtttca aggaagcagt tgtatgacgc tgtatcttcc aggataggac aaagattaca 300  
 ccagttaaag caacagggtg ataaagtatc tctgggagca caatatteag aagaatcacc 360  
 agcccttctt ggtaaacaaa tttcccaaac cgatgtncgt ctcaatgggt tggactggca 420  
 tgttcctaaa agggattgcc atccgaagga agcatcagcc tgaaggttaa gaggtttatg 480  
 35 gactctacag 490

&lt;210&gt; 20

&lt;211&gt; 386

&lt;212&gt; DNA

40 &lt;213&gt; Zea mays

&lt;220&gt;

&lt;221&gt; misc\_feature

45 &lt;222&gt; (1) .. (386)

&lt;400&gt; 20

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 tcagccgatt agaagattga agtggggaac ggccagtcct attgtccgag cacctataac 120  
 50 tccaatagtt ttgccaatg ttcactctgg tttcgaaaag gtcatgccag aaaactcggt 180  
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 gccaatagag tttgaettgc caagcttgaa gcaagaagca tcaacggtag cccatgactc 300  
 atcctctgaa cgggaagggt ggccggccat tacaccagat gggctggacg aggccgccc 360  
 55 gagatggcct taccagaaga tgtcag 386

&lt;210&gt; 21

&lt;211&gt; 429

&lt;212&gt; DNA

15

&lt;213&gt; Brassica napus

&lt;220&gt;

&lt;221&gt; misc\_feature

5 &lt;222&gt; (1) .. (429)

&lt;400&gt; 21

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agcagggaat ggacatcgcg acgtcgaat tgaataatgg aggatgggtt cacatatctc 360  
cagaaggcag acgggtaccga gatggtggct agactatggg ttcaacgcaat agaggtattg 420  
15 gaatgttgt 429

&lt;210&gt; 22

&lt;211&gt; 436

20 &lt;212&gt; DNA

&lt;213&gt; Brassica napus

&lt;220&gt;

&lt;221&gt; misc\_feature

25 &lt;222&gt; (1) .. (436)

&lt;400&gt; 22

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30 tttccgcact gacacgcgta tacctataac tagagggtgg ggaatctacc aagaacacat 180  
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ccaatcgctc tgccaataat tcaccgtggt tetgacgaga tgatgccgga gaagtacatc 360  
tatgggaagaa taccaccgtt accgctgtgg aacaaaaacc ttaagtagt tgttgggtga 420  
35 ccaatcagag ttgatg 436

&lt;210&gt; 23

&lt;211&gt; 423

40 &lt;212&gt; DNA

&lt;213&gt; Brassica napus

&lt;220&gt;

&lt;221&gt; misc\_feature

45 &lt;222&gt; (1) .. (423)

&lt;400&gt; 23

ggatgatcca tttgtcattg cttegttact tcgcctaag ettcactctg atgctcgtaa 60  
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50 ettcgatcc gtcaagggtt tgccagtctc tcgcggtgaa ggaatttate agcagggaat 180  
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tttggacgca gataccctcc ctaatgttgt tccatttgtg catactggta tgcaagatat 360  
aatgectata ggagccagtg ttccacggat tggcaaaaaca gtgacagtga tcactggaga 420  
55 tcn 423

&lt;210&gt; 24

&lt;211&gt; 400

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<212> DNA  
<213> *Oryza sativa*

<220>  
5 <221> misc\_feature  
<222> (1)..(400)

<400> 24  
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ccaagagagg tgttgggaaga ttggtaatgg acgctgacag ccttcagtt gtaataccct 180  
ttgtccatac aggaatgcag gatataatgc ctgtcggaaa acgtattcca agagcaggca 240  
aaaggggtgat tgrgggttgtt ggtgatccaa tcaacttcaa cgaccttacc attgacaaca 300  
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ggcagagact gcagggaactg aaagccgaag tcgatagatt 400

<210> 25  
20 <211> 414  
<212> DNA  
<213> *Brassica napus*

<220>  
25 <221> misc\_feature  
<222> (1)..(414)

<400> 25  
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17

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 <213> Brassica napus

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25 &lt;400&gt; 34

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&lt;210&gt; 35

&lt;211&gt; 449

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&lt;213&gt; Brassica napus

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45 &lt;400&gt; 35

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aggtggacgc tntgtgctac agatagatg
449

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55



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16. April 2003

**EP- Erstanmeldung**

**54 384 /X**

**Teil I**

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16. April 2003

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